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Our goal is to induce a strong $CD4^+$ T cell response against tumor antigens by preferentially presenting endogenous tumor antigens via class II major histocompatibility complex molecules (MHC II). MHC II can present endogenous tumor antigens if expressed in the absence of Invariant chain (Ii). We have up-regulated MHCII and down regulated Ii without affecting MHC II expression in tumor cells. Using the key transcription factor class II trans-activator (CIITA) we have coordinately up-regulated all class II MHC molecules (DR, DP, DQ) and associated molecules such as the Invariant chain in a Human mammary carcinoma (MCF10). We have successfully down regulated the invariant chain in MCF10 cells, up regulated for MHC II, using retroviral vectors that express siRNAs as hairpin loops. Immuno-fluorescence shows no down regulation of MHC II molecules on the cell surface after Ii was down regulated. We will test the ability of our vaccine to present tumor antigen by observing whether these cells can stimulate HER2/neu restricted $CD4^+$ or $CD8^+$T cells. These tumor cells could be used as a vaccine stimulating both $CD4^+$ and $CD8^+$ T cells in close proximity inducing a powerful long-term immune response against tumor sharing common tumor antigen with the vaccine.

14. SUBJECT TERMS
Immunotherapy, class II MHC, $CD4^+$ helper T lymphocyte, Invariant chain, siRNA, RNA

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Table of Contents

Introduction:

It may be possible to aid the bodies own immune system to better recognize and reject tumor by using genetically modified, irradiated whole tumor cells as a vaccine. This grant focuses on up regulating class II major histocompatibility molecules (MHC II) in order to facilitate activation of Helper T cells and down regulating the class II associated invariant chain (Ii), a molecule known to interfere with a cells' ability to alert helper T cells to foreign or malignant proteins in its repertoire of endogenously synthesized peptides. Human breast cancer tumor cells up-regulated for MHC II, down-regulated for Ii, and co-expressing costimulatory molecules such as CD80, may preferentially present endogenously encoded tumor antigens and efficiently activate tumor-specific $CD4^+$ T lymphocytes (Helper T cells). Human tumor cell lines can be up regulated for MHC II by expressing a transcription factor CIITA (Class II trans-activator) and down regulatated for Ii using RNA interference. The proposed tumor vaccine may also directly activate tumor specific $CD8^+$ T cells providing an ideal environment for T cell activation in which both activated $CD4^+$ and $CD8^+$ T cells are in close proximity to each other. This presentation scenario where $CD8^+$ and $CD4^+$ cells are stimulated to tumor antigen in close proximity could give optimal "Help" to CD8+ T cells, thereby providing a strong immune response and long-term immunity to tumor. Irradiated, genetically modified cells should function as cell-based vaccines, which will be tested for their ability to activate syngeneic peripheral blood mononuclear cells (PBMC), and to break tolerance and/or activate T cells from tumor-bearing individuals. These cells will also be tested with specific T cell lines restricted to their haplotype and recognizing a known tumor antigen to prove that these cells can act as antigen presenting cells to activate a tumor specific response. These studies will provide useful information on the role of tumor cells as antigen presenting cells that activate or anergize host T lymphocytes in breast cancer patients, and may provide a powerful tool for activation of the immune system against primary tumor and metastatic disease. In this project, I will test this hypothesis using human breast cancer cells and PBMCs as well as the transplantable mouse 4T1 tumor system.

Body:

Technical Objective 1: Develop siRNA for Ii and test its ability to down-regulate Ii in human and mouse breast cancer cells that constitutively express MHC II or are induced by IFNγ to express MHC II.

***Task 1**: Months 1-2: Use flow cytometry to ascertain that the human mammary carcinoma cell line SUM159 and the mouse mammary carcinoma 4T1 express MHC class II when induced with IFNγ.*

Upon typing it was found that SUM 159 did not contain a common allele of MHC II and would not be the ideal model for breast cancer. We set out to identify breast cancer cell lines that did not constitutively express MHC class II genes and, upon induction, expressed one of the common HLA-DR allele in use currently by this lab. These cells

needed to express MHC complex genes, including DR and Invariant chain (Ii) upon either Interferon gamma (IFN-γ) induction or stable expression of the Class II trans-activator (CIITA). Because of the diverse functions of IFN-γ in activating a wide array of genes, we have decided that using CIITA directly to activate MHC II may be preferential to avoid activating other genes that could affect the outcome of an experiment.

The Breast epithelial adenocarcinoma line MCF10ACA1 (hereto referred to as MCF10) was found to be a good candidate for our model [3]. I have shown by flow cytometry that the MCF10 cell line does not constitutively express HLA-DR or Ii (figure 1). Typing of the donors PBMCs, kindly provided by Wei-Zen Wei at the Karmanos Cancer Institute, has shown that one of its HLA-DR alleles is DR0701 a common allele found in roughly 13.28% of the North American Caucasian population [4]. These cells are easily transduced by retrovirus having 30 to 35% transduction efficiency, as observed in this lab. MCF10 cells were not responsive to low levels of IFN-γ but were inducible for the MHC II complex when transduced with retrovirus carrying a retroviral vector expressing CIITA (Fig 3). Another benefit to using the MCF10 cell line is that it expresses a known, well classified, tumor antigen HER2 (human Epithelial Receptor 2) shown by flow cytometry (Fig2). This can act as a model antigen to test whether this tumor model can present endogenous tumor antigen to activate $CD4^+$ T cells specific for HER2. I may also be able to test the vaccine's ability to directly stimulate $CD8^+$ T cells specific for HER2. Both $CD4^+$ and $CD8^+$ HER2 specific cell lines exist and are available [5]. There is a T cell clone that recognizes HER2 (776-788) in the context of HLA-DRB1*0701 [6]. Cells were also transduced with virus expressing the model antigen Tetanus toxoid for use as a model antigen in in-vitro T cell activation studies. TT expression is shown by immunofluorescence using flow cytometry (Fig. 1).

***Task 2**: Months 2-4: Design 21bp DNA oligonucleotides complimentary to 15 different sites on the Human Invariant Chain (Ii) sense and antisense strands. Anneal the strands together and Make siRNA in vitro with T7 RNA Polymerase.*

Complementary sequences in the coding region of the Human and Mouse Ii were chosen using the Ambion siRNA target finder search engine. Sequences with no homology to other known human mRNAs were chosen at random covering regions from the 3' to 5' end of the Ii mRNA. siRNAs were produced by in vitro transcription with T7 RNA polymerase as done by Yu et al [43] using the Ambion Silencer™siRNA Construction Kit (Fig.5a).

***Task 3**: Months 3-8: Test individual siRNAs for effective down-regulation of Ii in breast cancer cell lines described in Task 1.*

Because of the low transfection efficiency of the MCF10 cell line and many other human cell lines I opted to first look for functional siRNA sequences in a model cell line. 293T cells were chosen due to their high transfection efficiency using inexpensive transfection methods such as calcium phosphate transfection, which yields up to 90% transfection efficiency. The CIITA retrovirus was used to transduce the 293T cell lines, which were

then sorted for HLA-DR expression by magnetic-bead cell separation MACS® (Miltenyi)[7, 8] and shown to be HLA-DR$^+$ and Ii$^+$ by flow cytometry (Fig. 4). siRNAs made in-vitro were used to transfect the 293T/CIITA cells and Ii expression was determined by intracellular imunofluorescence (Fig 6). The Human Ii siRNAs 4 and 50 showed promising down regulation of Ii shown by flow cytometry. Because internal staining for Ii using Pin1 antibody gives some non-specific shift in fluorescence I compared the staining of our experimentals to the negative control 293T staining and to the positive control staining of 293T/CIITA (Fig. 6).

***Task 4**: Months 3-8: Clone Human CD80 cDNA into pLPCX retroviral vector (Clontech).*

CD80 was cloned into pLPCX and pLHCX [9]. Paper is attached as Appendices II.

***Task 5**: Months 6-10: Clone Human U6 RNA Polymerase III promoter with a multicloning site downstream into retroviral vector pLPCX/CD80 from Task 4.*

We opted to use a separate vector to express the siRNAs because of our observations made in task 7 that the retroviral vector 3'LTR promoter interferes with the U6 promoter function. We may pursue making a bicistronic vector expressing CD80 and siRNA down regulating Ii at a later time.

***Task 6**: Months 8-12: Clone DNA oligonucleotide coding for the sense-loop-antisense of successful siRNAs downstream of U6 promoter in pLPCX/CD80/U6 from Task 5. Clone DNA oligonucleotide coding for control siRNA (Lamin A/C) described by Paul et al (Paul, 2002 #23).*

The sense and antisense strands representing the functional siRNAs made in-vitro were cloned into the siRNA expressing retroviral vector pSIREN-RetroQ separated by one of two different loop structures, one recommended by Ambion the other recommended by Clontech. A full description of the making of siRNA expressing retrovirus is given in task 9. Positive control vectors were made but not used in vivo due to the positive results obtained by using the siRNA directed toward Ii. Negative control siRNA expressing vectors were made using sequences shown to not down regulate Ii in task 3.

***Task 7**: Months 10-14: Transfect packaging cell line (293T Human Embryonic Kidney cells) with pLPCX/CD80/Ii$^-$ retroviral vector from Task 6. Harvest virus and titer on NIH3T3 mouse fibroblasts.*

Virus was successfully made and titered on MCF10/CIITA cells and found to infect cells at 30-70% efficiency.

***Task 8**: Months 13-16: Transduce human SUM159 and mouse 4T1 mammary carcinoma cells with retrovirus encoding CD80 and siRNA for Ii and select transductants by drug selection. Limit dilution clone if necessary.*

MCF10 cells were used in place of SUM159 as explained in task 1. MCF10/CIITA cells and MCF10/CIITA/CD80 cells were transduced with various siRNA expressing retrovirus, which is drug selectable using puromycin. Because CD80 is drug selectable by hygromycin we were able to drug select for cells that expressed CD80 and siRNA for Ii by growing cells in puromycin and Hygromcyin. 4T1 cells have not been transduced with the siRNA expressing retrovirus as I need to design and test siRNAs to specifically down regulate the mouse Ii. The siRNAs designed for human Ii are not complimentary to the mRNA of the mouse Ii and will therefore not function in mice. I am currently designing and cloning siRNA expressing vectors specific for mouse Ii.

***Task 9**: Months 16-24: Induce cells generated in Task 8 with IFNγ. Use flow cytometry to verify MHC II and CD80 expression and functionality of the siRNA to down regulate Ii.*

Functional siRNA sequences were used to make siRNA expression cassettes using Ambion's Silencer™ Express siRNA Expression Cassette Kits (Fig. 5b) [10, 11]. The siRNA cassettes were digested with *EcoRI* and *HindIII* and cloned into the pLPCX retroviral vector. The CMV promoter of this vector was excised by digesting with *NcoI* to prevent any possible promoter interference or interference from antisense RNA driven by the CMV promoter. Retroviruses containing this vector were used to transduce MCF10/CIITA cells. Initially the siRNA4 vector showed some down regulation of Ii by flow cytometry on day 2 after infection (Fig. 7) but by day 5, the effect was not detectable (Data not shown). Encouragingly I saw no down regulation of MHCII molecules on day 2, when Ii was down regulated (Data not shown). This is important because it was previously believed that class II MHC could not be expressed on the cell surface without Ii due to observations made in mouse Ii knockout studies showing markedly decreased surface expression of class II MHC in these mice [12]. I believe that our original siRNA retroviral expression vector may not be stable because of promoter interference, due to the 3'LTR having promoter activity [13]. In addition, in a comparative study done by Ilves et al. they found that pol III promoters, such as the U6 promoter I am using, are not active when placed in between the 5' and 3' LTRs and were only able to express their gene when the promoter was placed in the *NheI* site of the 3' LTR [14]. I also believe the siRNA 50 virus was not functional because it contains a four consecutive thymine stretch that could have acted as a premature stop of transcription by the U6 promoter. I have therefore decided to use the Clontech pSIREN-RetroQ vector which has the RNA polymerase III U6 RNA promoter to stably express siRNA (Fig. 8) [10, 15-18]. It also has a portion of the 3'LTR excised to avoid promoter interference and has been shown to stably express siRNA in mammalian cells. To avoid premature termination of transcription in siRNA50 I have identified sequences before and after that region of the Ii mRNA and designed oligonucleotides to express the appropriate siRNAs. The siRNA hairpin sequences were cloned into the Clontech vector pSIREN-Retro-Q and used to make virus. The siRNA expressing retrovirus was used to infect MCF10/CIITA and showed to functionally down-regulate Ii at least two weeks after transduction (Fig 9 and 11a). Interestingly two of these siRNAs seem to have up regulated the Ii (Fig 9 and 11a siRNA 4.2 and 54). I have shown by immunofluorescence

and Western analysis that in cell lines down regulated for Ii by over 95%, MHC II is still expressed stably at the cell surface (Fig 10 and 11b).

Technical Objective 2: As a model system in which to test "proof of principle" we will test the siRNA approach in vivo in mice with metastatic mammary carcinoma.

***Task 10**: Months 20-24: Inoculate naïve BALB/c mice with genetically modified 4T1 cells and follow for survival and quantify number of metastatic cells in the lungs, liver, bone marrow, and brain using the clonogenic assay.*

Not yet accomplished. On schedule.

***Task 11**: Months 24-30: Inoculate BALB/c mice with wild type 4T1 tumor cells and allow primary tumors to metastasize. Surgically remove primary tumors and treat mice with irradiated retrovirally transduced $CD80^{+}Ii^{-}$ mouse 4T1 cells that have been in vitro treated with IFNγ. Follow mice for survival and quantify the numbers of metastatic cells in distant organs per task 10.*

Not yet accomplished. On schedule.

Technical Objective 3: Test MHC II^{+} $CD80^{+}$ Ii^{-} human breast cancer cells for their ability to activate breast-cancer-specific $CD4^{+}$ T cells from PBMC of tumor-bearing individuals.

***Task 12:** Months 18-22: Using primary tumor for which autologous PBMC are available (provided by our collaborator, Dr. A. Stopeck), identify primary breast cancer cells, which are IFNγ inducible or constitutively express MHC II.*

Not yet accomplished. On schedule. We are collaborating with Dr. Keith Knutson who is making $CD4^{+}$ T cell lines restricted to DR7 and specific to HER2/neu, which we plan to use to show that MCF10 cells positive for MHC II, CD80 and negative for Ii can present a known endogenously expressed tumor antigen (HER2/neu) to activate CD4+ T cells. We also have genetically modified MCF10 cells to express the fragment C of the Tetanus toxin which will be used as a model antigen in vitro (figure 1). We have previously stimulated T cells in PBMCs with fragment C of tetanus toxin (TT) to make TT specific T cells [9]. There is a known DR7 specific epitope in fragment C of TT [19]. We can use these T cells to test whether MCF10 expressing MHC II (one allele known to be HLA-DR7) and down regulated for Ii can present an endogenous model antigen to $CD4^{+}$ T cells. This data together with the mouse studies proposed in technical objective 2 should be sufficient to merit clinical studies with this tumor vaccine. We have made MCF10 cells that express only the HLA-DR7 MHC II allele as a control to differentiate between a specific response to antigen in context of MHC II from an allogeneic response to different MHC II alleles on the tumor cells compared to T cells in PBMCs of hosts that are only matched for HLA-DR7. In order to express only the HLA-DR7 we had to correct a mistake in the existing DR7 cDNA in our possession using splicing by

overlapping extensions (SOEing) (Fig. 12). The corrected HLA-DR7 beta cDNA was cloned into the pLNCX2 retroviral vector containing the alpha subunit and an internal ribosomal entry site as described in Dissanayake et al. (Fig 13) [9]. I also cloned the HER2/neu gene into the pLPCX retroviral vector for increased expression of HER2/neu in MCF10 cells in case the existing level is not sufficient to present endogenous antigen to T cells (Fig 14). Cells expressing HLA-DR7 and HER2/neu were screened by immunofluorescence using flow cytometry (Fig 15).

***Task 13**: Months 22-25: Transduce the primary human breast cells identified in Task 12 with the CD80$^+$ siRNA retrovirus. Induce cells with IFNγ, and use flow cytometry to ascertain expression of MHC II and CD80, and lack of Ii expression.*

Not yet accomplished. On schedule.

***Task 14**: Months 25-32: Perform antigen presentation assays using autologous PBMCs as responding lymphocytes and retrovirally-modified autologous breast cancer cells from task 13 as antigen presenting cells. Quantify T cell activation by measuring IL-2 production by ELISA.*

Not yet accomplished. On schedule.

***Task 15**: Identify the responding T cells in Task 14 by blocking MHC class I or II and/or by depleting CD4 or CD8 T cells before setting up the antigen presentation assays. Ascertain the type of CD4 response (Th1 or Th2) by assessing IL-4 and IFNγ production by ELISA and in situ cytokine capture.*

Not yet accomplished. On schedule.

Key Research Accomplishments:

- **Up regulated all class II MHC complex molecules in the human breast cell line MCF10 by expressing CIITA via a retroviral vector.**
- **Successfully expressed costimulatory factor CD80 in the human breast cell line MCF10 via a retroviral vector.**
- **Successfully identified four functional siRNAs for the down regulation of Human Ii.**
- **Have made retrovirus that are capable of delivering and expressing small hairpin RNAs that down regulate Human Ii for over two weeks and show no signs of promoter interference.**
- **Successfully down regulated Human Ii in the tumor cell line MCF10 by over 95% using RNA interference.**
- **Have made control cell lines expressing only one MCH II allele of HLA-DR7 as well as a model antigen Tetanus Toxin fragment C.**

Reportable Outcomes:

- Dissanayake SK, Thompson JA, Bosch JJ, Clements VK, Chen PW, Ksander BR, Ostrand-Rosenberg S. Activation of tumor-specific CD4(+) T lymphocytes by major histocompatibility complex class II tumor cell vaccines: a novel cell-based immunotherapy. Cancer Res. 2004 64:1867-1874.
- Presented poster at American Association of Immunologists (AAI) Experimental Biology 2004, April 17 – 21, WASHINGTON, DC. **Abstract Title**: Enhanced presentation of endogenous MHC class II-restricted peptides by tumor cell vaccines via RNAi-mediated down-regulation of Invariant chain. **Poster Session Title**: Cancer Vaccines and Immunotherapies. **Day of Presentation**: April 18
- Presented poster at 2004 AACR (American Association of Cancer Researchers) Annual Meeting in Orlando, Florida. **Abstract Title**: Novel MHC class II breast cancer vaccine using RNA Interference (RNAi) to down regulate invariant chain. **Session ID**: Immunology/Tumor Immunobiology 9. **Session Date and Start Time**: Tuesday, March 30, 2004, 1:00 PM
- Spoke at University of Maryland Graduate Student Association of Biological Sciences (GABS) Symposium 2004.

Conclusions:

I have successfully up regulated MHC II molecules and down regulated the invariant chain in a Human breast cell line. It is clear now that Ii is not necessary for stable expression of MHC II at the cell surface of tumor cells. I will now show that these cells can present endogenous tumor antigens to CD4+ T cells activating them. This will be done by in vitro assays with an HLA-DR7 restricted T cell line specific for HER2/neu expressed by this cell line. I will also make a DR7 restricted Tetanus Toxin specific T cell line for in vitro assays with tumor cells expressing the fragment C of Tetanus toxin, to show that this phenomenon is not antigen specific.

In vitro studies using the mouse 4T1 model are to follow, showing if tumor cells genetically modified to express MHC II and down regulated for Ii can functiona as a vaccine, reducing metastasis.

Together these studies will provide a strong strategy to induce a long term immune response in patients with a risk of metastatic disease such as is the case in breast cancer patients.

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Appendices I: Figures

Fig 1

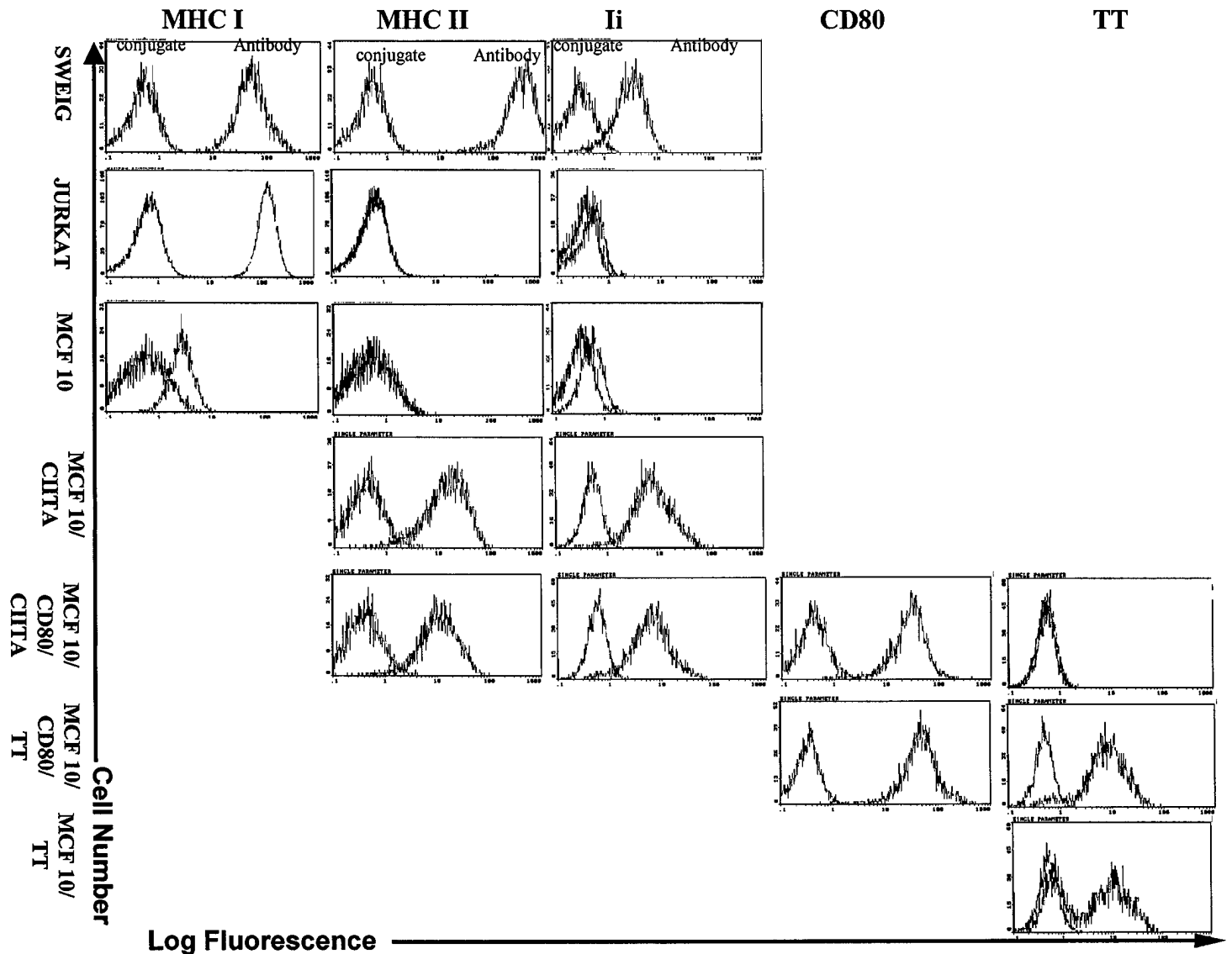


Figure 1. MCF10 cells do not constitutively express HLA-DR or Invariant chain (Ii), but DR and Ii are up regulatable by CIITA. This cell line was genetically engineered to express CIITA (inducing DR and Ii expression), CD80 (a costimulatory molecule) and fragment C of Tetanus toxoid (a model antigen). Live transductants were stained by indirect immunofluorescence with L243 (HLA-DR), CD80 antibody (CD80) or W6/32 (HLA-class I). Fixed cells were stained by indirect immunofluorescence for internal PIN1.1 (Ii) and TT monoclonal antibody (Tetanus Toxoid). Control cell lines: SWEIG(+ve control) is a human B cell lymphoma and Jurkat (-ve control) is a leukemic T cell line. All samples in this study were analyzed using an Epics XL flow cytometer. Blue peaks denote background staining with conjugate antibody alone and Red peaks denote staining with both primary and secondary antibodies.

Fig 2

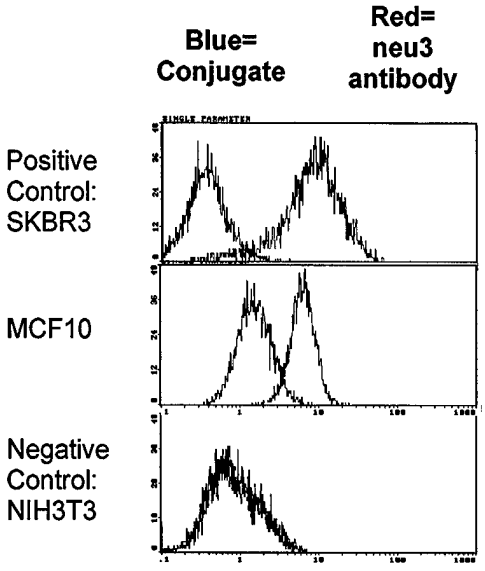


Figure 2. MCF10 express the tumor antigen HER2. Live transductants were stained by direct immunofluorescence for plasma membrane HER2 (neu3 antibody). Blue peaks denote staining with fluorescent conjugate alone; red peaks represent staining with primary antibody plus fluorescent conjugate.

Fig 3

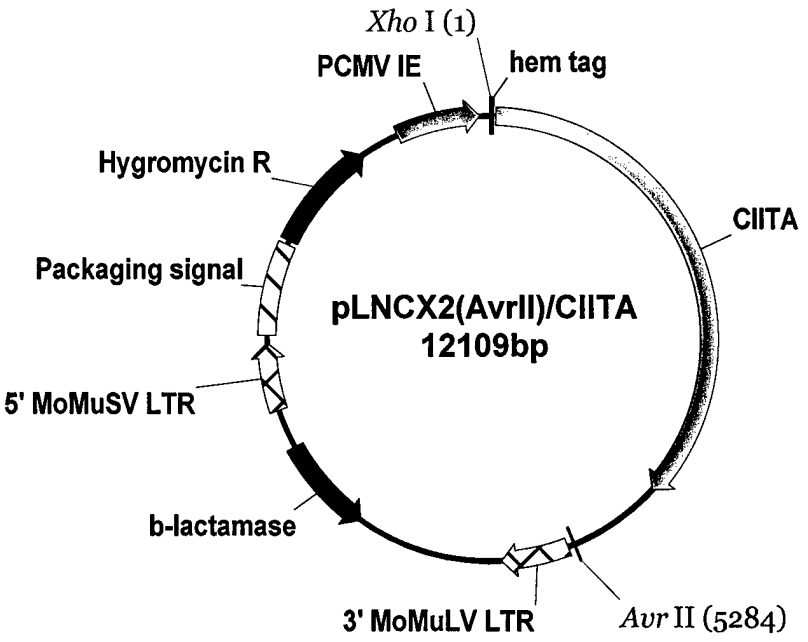


Figure 3. pLNXC2 (AvrII)/CIITA construct.
A linker containing an *Xhol* site was cloned into the *Spel* and EcoRI restriction digested pcDNA1amp-tag/CIITA vector upstream of the cDNA for CIITA. The linker sequences were: CTAGTGTCTCTCGAGGAACG and AATTCGTTCCTCGAGAGACA.
The cDNA for CIITA was then cloned into the pLHCX-L vector with XhoI and AvrII.

Fig 4

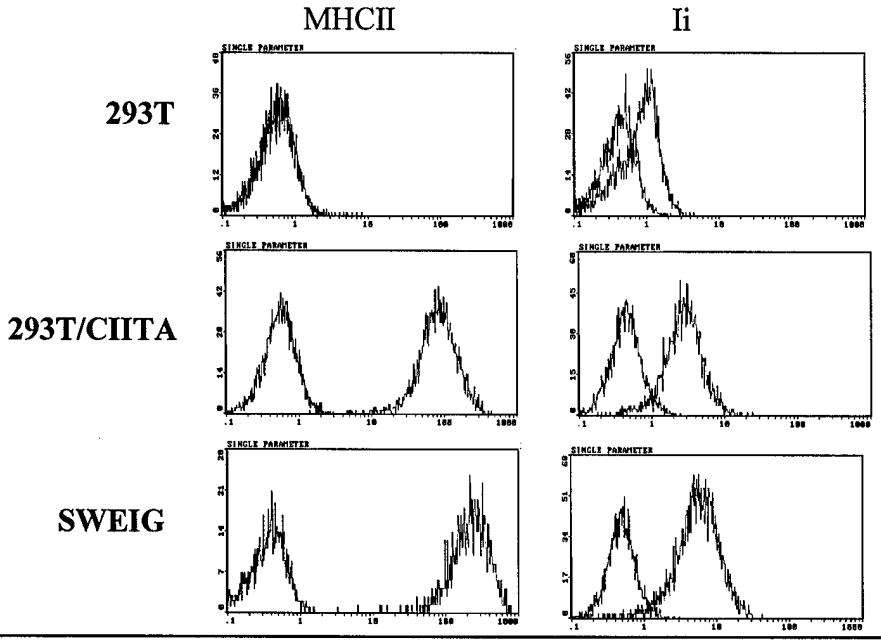


Figure 4. The parental line 293T cells do not express HLA-DR or Ii. 293T transductants expressing CIITA are up regulated for HLA-DR and Ii. Live transductants were stained by direct immunofluorescence for plasma membrane HLA-DR (L243-FITC). Fixed cells were stained by indirect immunofluorescence for Ii (mAb PIN1.1). Blue peaks are stained with conjugate alone and red peaks are stained with primary and conjugate antibodies.

Figure 5a

In Vitro Transcription of siRNAs

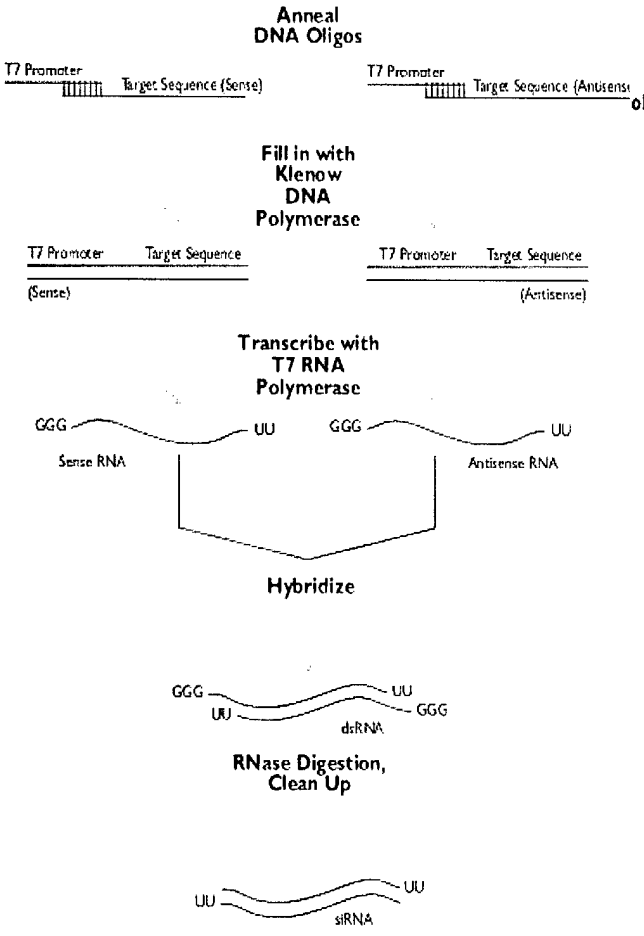


Figure 5b

Generating an siRNA Expression Cassette

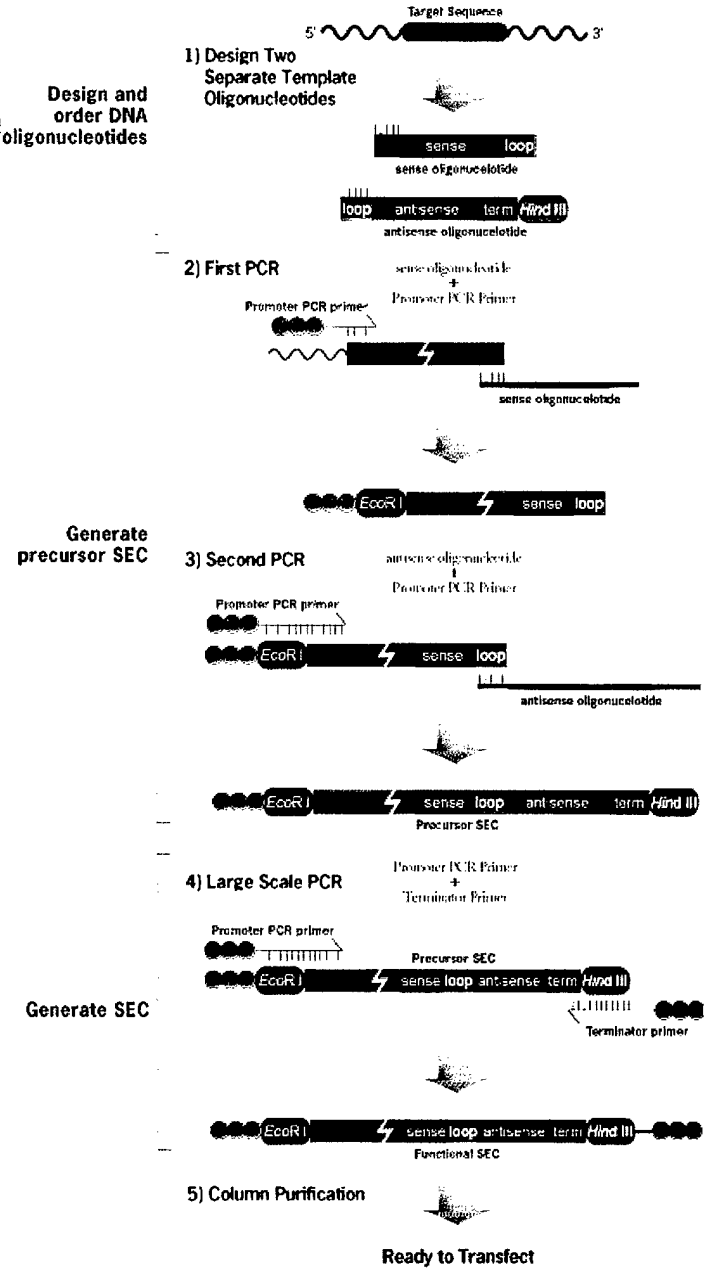
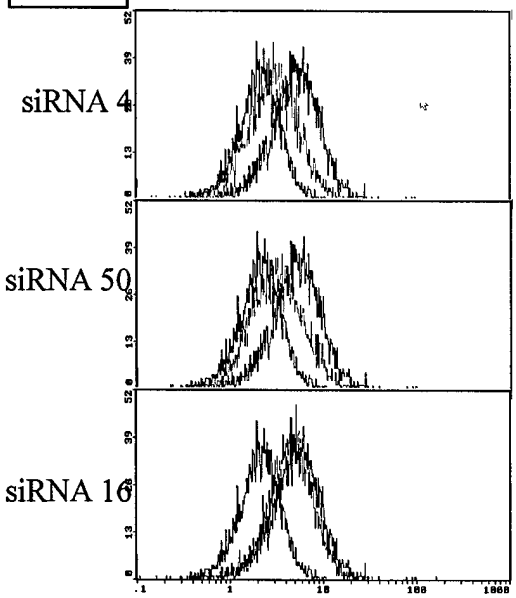


Figure 5a and 5b. Methods for making siRNA used in this study. 8a: In-vitro transcription using the T7 RNA polymerase to produce double stranded RNA. 8b: Construction of a siRNA-expressing Cassette driving expression of siRNA by a pol III promoter U6 by PCR. This linear double stranded DNA can express siRNA when used to transfect cells with or it can be incorporated into a vector system for delivery and more stable expression in cell lines.

Fig 6



Blue = 293T Pin1
Red = 293T/CIITA Pin1
Green = 293T/CIITA siRNA transfected Pin1

Figure 6. 293T/CIITA cells transfected with siRNA 4 and 50 had reduced expression of Ii compared to siRNA 16. Cells were transfected with siRNAs. Cells were fixed and stained with PIN1.1 monoclonal antibody. Cells transfected with siRNA 4, 50 or 16 are shown in row 1, 2, and 3 respectively. The blue peak is staining of parental 293T (no Ii) with Pin1.1 plus conjugate antibody. The Red peak is staining of 293T/CIITA (Ii expressing) with Pin1.1 and conjugate. The Green peak shows staining of transfectants with Pin1.1 and conjugate.

Fig 7

siRNA down regulation day 2 after transduction of MCF10/CIITA/siRNA

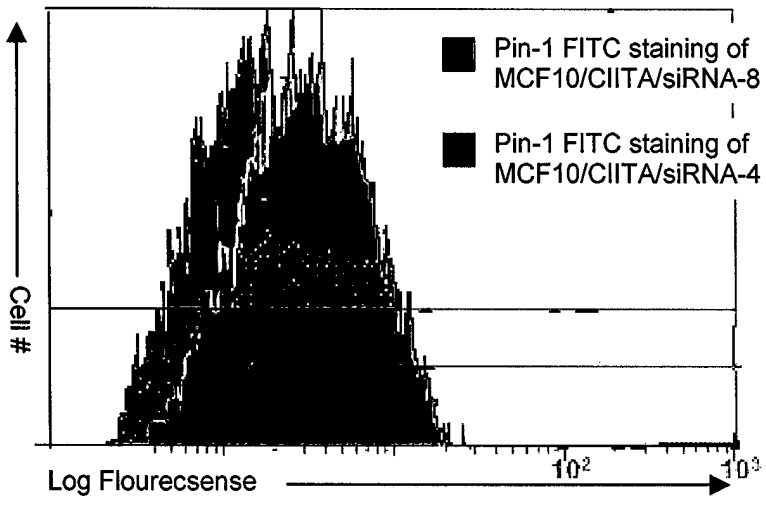


Figure 7. MCF10/CIITA transduced cells expressing HLA-DR7 and Ii were down regulated for Ii two days after transduction. Cells were transduced with virus containing the retroviral vector pLPCX/SEC 4, or pLPCX/SEC 8. After a week of selection for puromycin resistant cells the down regulation of Ii was no longer observed (Data not shown). This retroviral vector contained the siRNA expression cassettes using the U6 pol III promoter to drive siRNA expression. The cassette was cloned into the multiple cloning site of pLPCX just upstream of a CMV-Promoter, which was excised. Conjugate staining and staining of negative control pLPCX/SEC 8 were done but are not shown here.

Fig 8

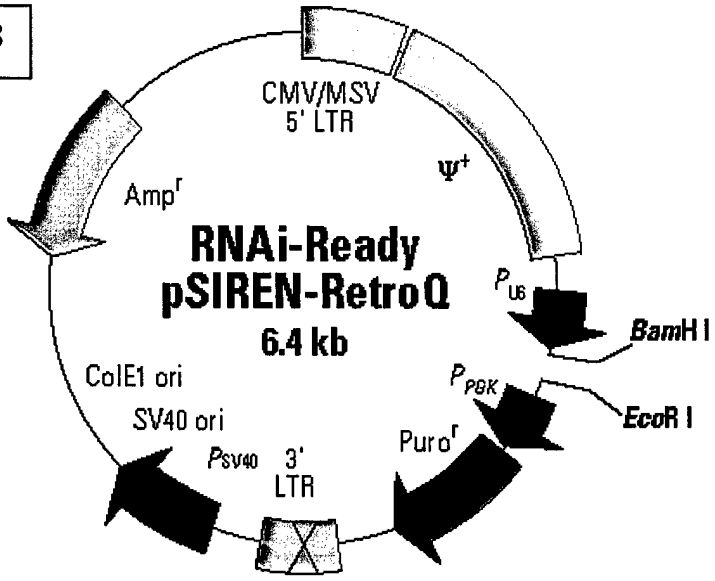
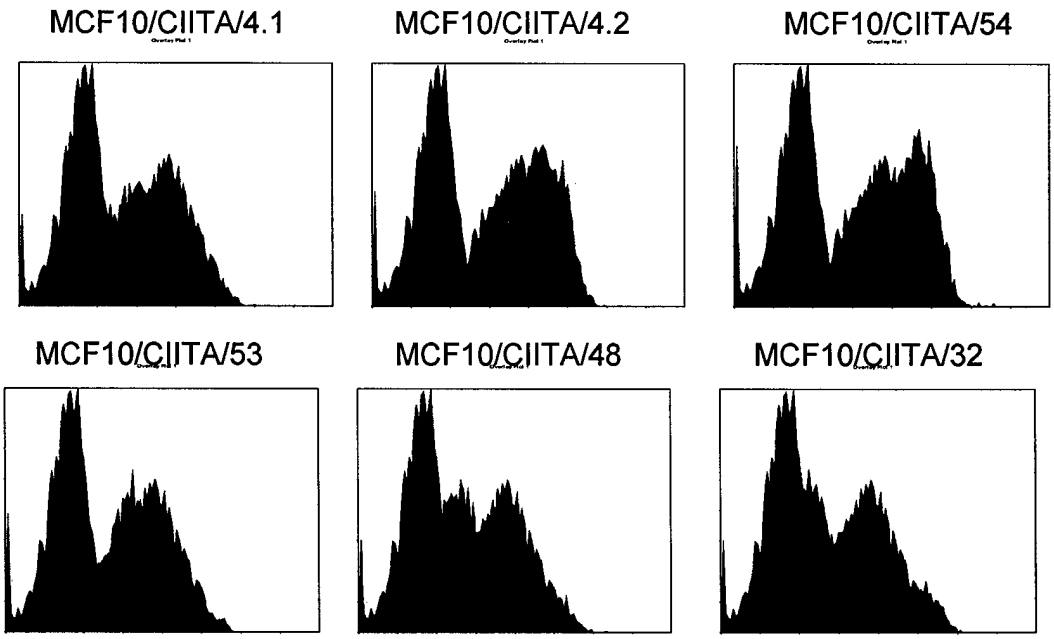


Figure 8. Retroviral vector commercially available from Clontec. SiRNA is expressed from a pol III promote with a well-defined start and stop of transcription. A portion of the 3' LTR was removed to prevent promoter interference. This vector has shown to stably express siRNA in mammalian cells such as MCF7.

Fig 9



Neg. Control MCF10 internally stained for invariant chain with Pin1
Pos. Control MCF10/CIITA internally stained for invariant chain with
Experimental: MCF10/CIITA transductants internally stained for invariant chain 2 weeks after transduction. (4-12-4)

Figure 9. Down regulation of Ii in MCF10/CIITA transduced with pSIREN-RetroQ vectors expressing small hairpin RNAs (shRNA) directed at Ii two weeks after transduction. Hairpin 4.1 and 4.2 differ in the stop sequence. siRNA 4.1, 53, 48 and 32 all showed to down regulate Ii. 4.2 and 54 up regulated Ii.

Fig 10

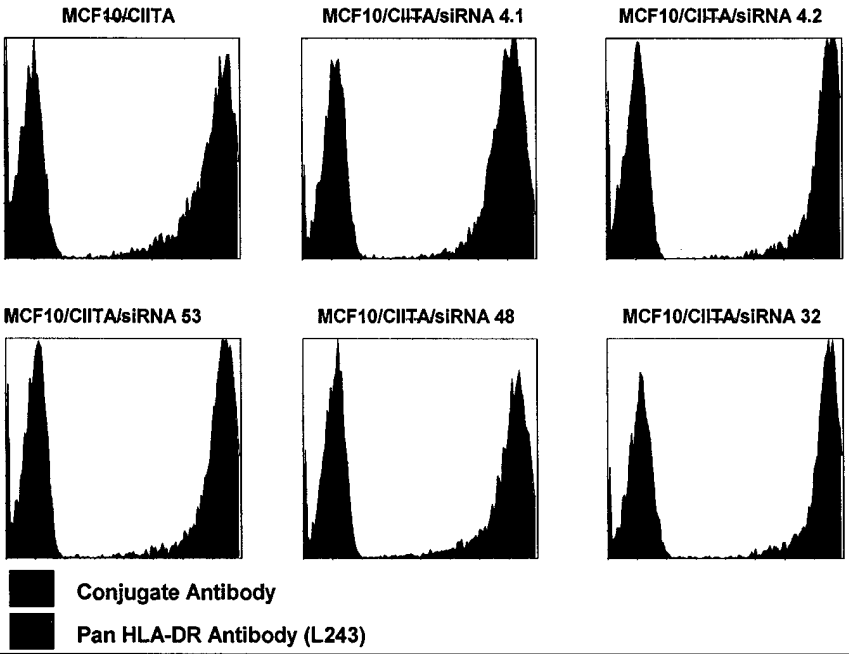


Fig 10. Staining of MCF10/CIITA/siRNA Ii for MHC II shows no significant decrease in stable surface MHC II. Live transductants were stained by indirect immunofluorescence with L243 (HLA-DR).

Fig 11a

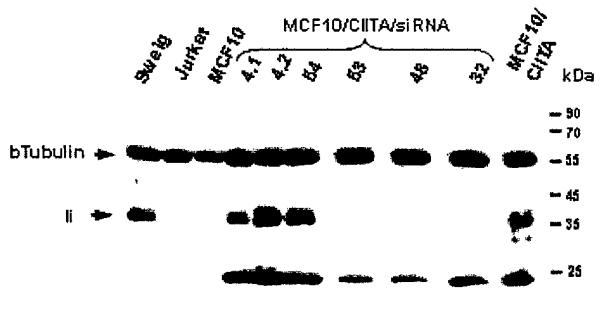


Fig 11b

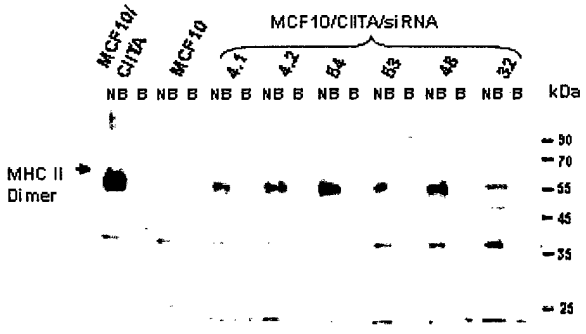


Fig. 11. Positive control Sweig is a B cell lymphoma positive for MHC II and Ii, negative control Jurkat is a leukemic T cell line negative for MHC II and Ii, MHC/CIITA are MHC II^+ and Ii^+, MCF10 are MHC II^- and Ii^-.

11a. Western analysis using primary Monoclonal Anti-b-Tubulin antibody produced in mouse Clone TUB 2.1 Ascites fluid (Sigma) as a control and monoclonal Anti-Ii antibody Pin1.1 [1] By Western MCF10/CIITA cells expressing siRNA 53 and 48 have no detectable Ii.

11b. Western analysis using Anti-DR mAb from the hybridoma cell line L243 [2]. Cell lysates were run in non denaturing conditions with 0.2% SDS and either not boiled (NB) or boiled (B).

Fig 12

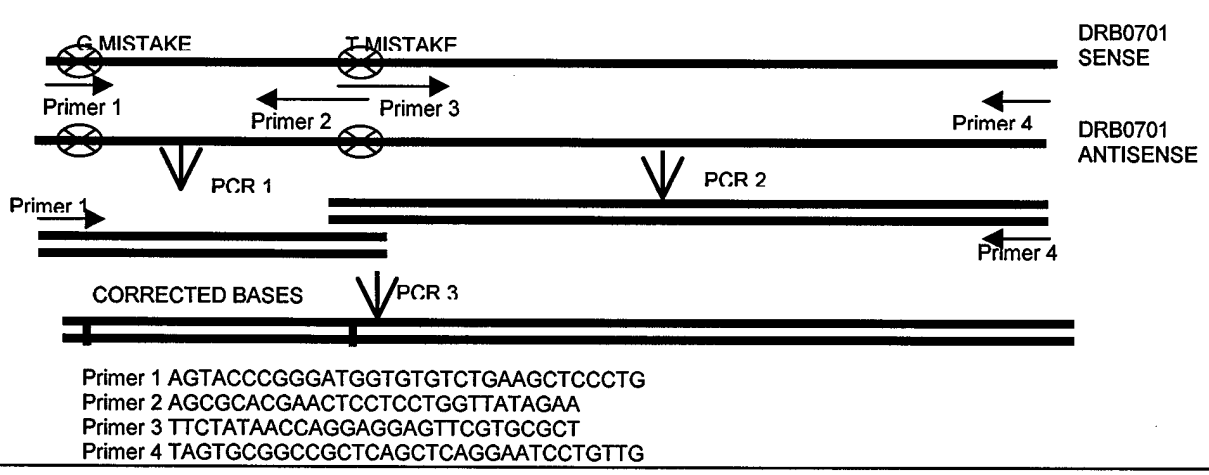


Figure 12. PCR1 rxn. Conditions: 95°C 2min, then 30 rounds of 95°C 30sec, 60.2°C 30sec, 72°C 1min. then 72°C for 10min. PCR2 rxn. Conditions: same as PCR1 but annealing temperature was 62.3°C. SOEing (PCR3) rxn. Conditions: 95°C 2min, then 5 rounds of 95°C 30sec, 60.2°C 30sec, 72°C 1min. Then 5 rounds ore of the same with annealing temp. at 62.3°C then 23 rounds of the same at 64.8°C then 72°C for 10min.

Fig 13

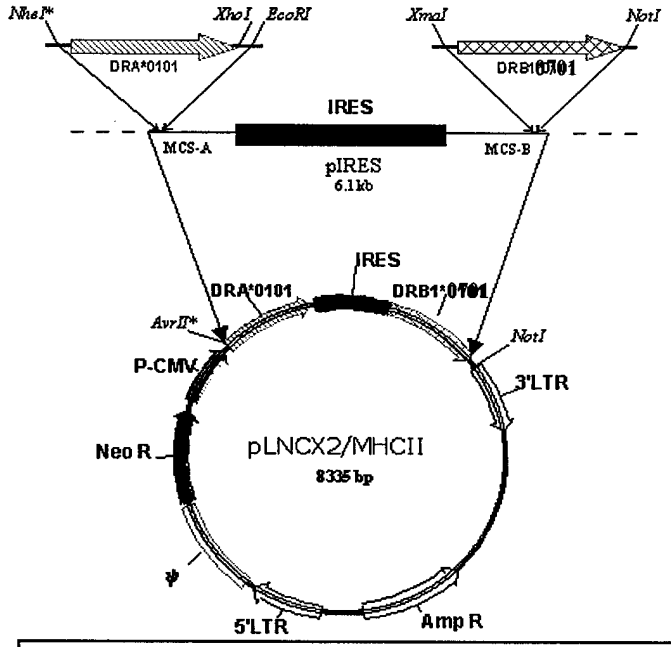


Fig. 13. The recombinant HLA-DR0101/pLNCX2 retroviral construct.
cDNAs encoding the HLA-DR1 alpha and beta chains were initially cloned into the MCSA and MCSB respectively of the pIRES vector (Clontec). The retroviral vector pLNCX2/MHCII was constructed by subcloning the DRA-pIRES-DRB segment of cDNA from pIRES vector to the pLNCX2 retroviral vector (Clontec) modified to include a linker containing an AvrII site in the MCS.

Fig 14

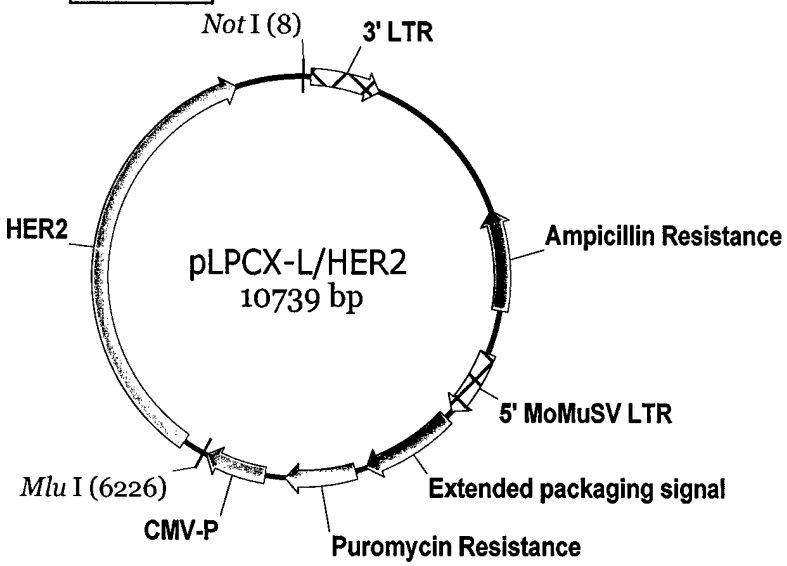


Fig. 14. pLPCX/HER2 vector. A linker containing the *MluI* and *AvrII* shown below was inserted into the pLPCX vector:
GATCTACGCGTCTCGAGAAGCTTGAATTCCCTAGGGC
GGCCGCCCTAGGGAATTCAAGCTTCTCGAGACGCGTA
The HER2 cDNA was cloned out of the pcDNAamp-tag/HER2 into the modified pLPCX-L. pcDNAamp-tag/HER2 was digested with *MluI* and *XbaI* and ligated to pLPCX-L digested with *MluI* and *AvrII*. The *XbaI* and AvrII have compatible cohesive ends. This site was lost upon ligation.

Fig 15

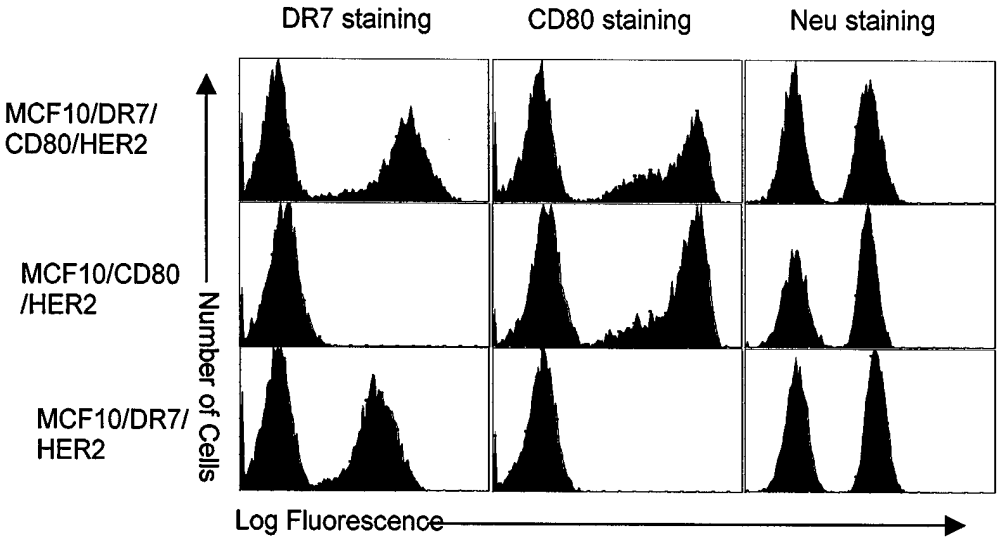


Fig. 15. MCF10 cell lines expressing DR7, CD80 and HER2 were made using retroviral expression vectors. Live cells were stained with L243 (HLA DR), CD80 antibody, or neu3 (HER2). Red peaks show staining with conjugate alone and Blue peaks shows staining with primary and conjugate antibodies.

[CANCER RESEARCH 64, 1867–1874, March 1, 2004]

Activation of Tumor-specific CD4$^+$ T Lymphocytes by Major Histocompatibility Complex Class II Tumor Cell Vaccines: A Novel Cell-based Immunotherapy

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ABSTRACT

Mouse tumor cells transfected with syngeneic MHC class II and costimulatory molecule genes are therapeutic vaccines in mice, provided they do not coexpress the class II-associated invariant chain (Ii). We demonstrated previously that the vaccine cells present tumor peptides via the endogenous antigen presentation pathway to activate CD4$^+$ and CD8$^+$ T cells. Because of their efficacy in mice, we are translating this vaccine strategy for clinical use. To obtain MHC class II$^+$CD80$^+$Ii$^-$ human tumor cells, we developed retroviruses encoding HLA-DR and CD80. The HLA-DR virus encodes the DRα and DRβ0101 chains using an internal ribosomal entry site to coordinate expression. SUM159PT mammary carcinoma and Mel 202 ocular melanoma cells transduced with the retroviruses DRB1/CD80 express high levels of DRB0101 and CD80 on the cell surface in the absence of Ii. Irradiated SUM159PT/DR1/CD80 vaccines stimulate proliferation of non-HLA-DRB0101 peripheral blood mononuclear cells and present an exogenous DR1-restricted tetanus toxoid (TT) peptide, indicating that the transduced DRB0101 is functional. SUM159PT/DR1/CD80 vaccines were further transduced with a retrovirus encoding the TT fragment C gene, as a model tumor antigen. These cells stimulate IFN-γ release from TT-primed human DRB0101 peripheral blood mononuclear cells, demonstrating their ability to present "endogenous" tumor antigen. Depletion and antibody blocking experiments confirm that MHC class II-restricted, endogenously synthesized epitopes are presented to CD4$^+$ T cells. Therefore, the MHC class II vaccines are efficient antigen-presenting cells that activate tumor-specific MHC class II-restricted, CD4$^+$ T lymphocytes, and they are a novel and potential immunotherapeutic for metastatic cancers.

INTRODUCTION

A key goal of cancer vaccine development is to generate therapeutic reagents that provide protection against development and outgrowth of metastatic tumor cells. Because metastatic disease for many tumors appears at varied intervals after diagnosis of primary tumor, the most effective vaccines will provide long-term immune memory. We (1, 2) and others (3–5) have focused on the critical role of CD4$^+$ T cells in cancer vaccines, because these cells, in conjunction with CD8$^+$ T lymphocytes, are likely to provide maximal antitumor immunity with long-term immunological memory.

To better activate tumor-specific CD4$^+$ T cells, we have designed cell-based vaccines that facilitate the presentation of MHC class II-restricted tumor peptides to responding CD4$^+$ T cells (2). We have reasoned that tumor cells present a variety of MHC-restricted peptides that are potential tumor antigens, and that if they constitutively express MHC class I molecules and are transduced with syngeneic MHC class II and costimulatory molecules, they could function as antigen-presenting cells (APCs) for MHC class I- and class II-restricted tumor peptides. This approach is appealing for several reasons: (*a*) identification of specific tumor antigen epitopes is not required; (*b*) multiple class I- and class II-restricted epitopes will be presented concurrently; and (*c*) CD4$^+$ T cells may be activated to novel MHC class II-restricted tumor epitopes not presented by professional APCs.

To test our approach, cell-based vaccines were generated from three independent mouse tumors that constitutively express MHC class I molecules and do not express MHC class II molecules (mouse SaI sarcoma, B16 melanoma, and 4T1 mammary carcinoma). The mouse tumor cells were transfected with syngeneic MHC class II α- and β-chain genes and with costimulatory molecule (*CD80*) genes. This vaccine approach was adapted for two reasons:

(*a*) In conventional immunity, activation of CD4$^+$ T cells requires the uptake of soluble antigen by professional APCs and the cross-presentation of the processed antigen to specific CD4$^+$ T cells. If antigen is limiting, as it may be when tumor burden is low, available antigen may not be sufficient for the activation of tumor-specific CD4$^+$ T cells. Our vaccine design bypasses the requirement for professional APCs and soluble tumor antigen because the genetically modified tumor cell vaccines function as the APC.

(*b*) Because each vaccine cell expresses both MHC class I and class II molecules and their associated tumor peptides, a given vaccine cell could be an APC for both MHC class I- and class II-restricted tumor antigen epitopes and concurrently activate both CD4$^+$ and CD8$^+$ T cells. If CD4$^+$ and CD8$^+$ T cells are simultaneously activated by the same APC and are in close proximity to each other, then the transfer of "help" from the activated CD4$^+$ to the CD8$^+$ T cell should be highly efficient (2, 6), thereby maximizing the therapeutic effect. CD4$^+$ T cell "help" could be provided to CD8$^+$ T cells via the classical mechanism of soluble cytokine production or by the alternative mechanism of up-regulation of CD40 on the vaccine cells (APCs; Ref. 7). Regardless of the mechanism of help, the activated CD4$^+$ T cells do not need to directly interact with wild-type tumor cells or with professional APCs, because their only role is to provide help to CD8$^+$ T cells.

Extensive studies using a variety of mouse tumor models have shown that immunization/immunotherapy with the MHC class II plus CD80-modified vaccines induces a potent antitumor immunity against wild-type tumor that confers prophylactic protection (1), delays or eliminates growth of primary solid tumors (8), reduces both experimental (9) and spontaneous metastasis, and extends survival (10, 11). Immunization studies using genetically marked vaccine cells have demonstrated that the vaccine cells themselves are the relevant APCs *in vivo* (12–14), and that both CD4$^+$ and CD8$^+$ T cells are required for the optimal antitumor effect (8, 11). Therefore, by circumventing the traditional cross-presentation pathway for activation of CD4$^+$ and CD8$^+$ T cells, these genetically modified cancer vaccines induce a potent tumor-specific immunity against wild-type tumor cells.

The efficacy of the vaccines depends on their ability to present endogenously synthesized, MHC class II-restricted tumor antigen epitopes to activate CD4$^+$ T cells. Presentation of endogenous antigen

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is favored when levels of the class II-associated accessory molecule, invariant chain (Ii), are limiting (reviewed in Ref. 15). Because MHC class II and Ii are coordinately regulated and Ii expression blocks vaccine efficacy (14, 16, 17), we have used tumor cells that do not constitutively express MHC class II (or Ii) as the "base" line for the vaccines.

Because of its therapeutic efficacy in mice, we are translating this vaccine approach for the treatment of human cancers. Our experimental design is to express syngeneic MHC class II and costimulatory molecules in established human tumor cell lines that constitutively express MHC class I molecules and do not constitutively express MHC class II or Ii. To achieve this goal, we are using retroviral transduction to express HLA-DR and CD80 molecules in two human tumor lines, an ocular melanoma (Mel 202) and a mammary carcinoma (SUM159PT). The resulting HLA-DRB0101-transduced cells stably express high plasma membrane levels of functional HLA-DRB0101, as measured by immunofluorescence, activation of allogeneic peripheral blood mononuclear cells (PBMCs), and presentation of a DR1-restricted peptide. To ascertain that the transductants activate $CD4^+$ T lymphocytes to endogenously synthesized antigens, we have shown that tetanus toxoid (TT) fragment C-transduced vaccine cells activate TT-specific HLA-DRB0101-restricted $CD4^+$ T cells. Therefore, human tumor cells genetically modified by gene transfer to express syngeneic MHC class II and costimulatory molecules express functional HLA-DR molecules and may serve as useful therapeutics for activating tumor-specific $CD4^+$ T lymphocytes of cancer patients.

MATERIALS AND METHODS

Construction of Retroviral Vectors. For the pLNCX2/DR1 construct, DRA cDNA in the RSV.5 vector (18) was PCR amplified including 5′ *Nhe*I and 3′ *Xho*I restriction sites: DRA 5′ primer, TGTCGCTAGCATGGCCATAAGTGGAGT; and DRA 3′ primer, ACTGCTCGAGTTACAGAGGCCCCCTGCGTT. The PCR product was cloned into the pCR2.1-TA vector (Invitrogen, Carlsbad, CA), excised with *Nhe*I and *Eco*RI, and inserted into the multiple cloning site (MCS)-A of *Nhe*I- and *Eco*RI-digested pIRES plasmid (Clontech, Palo Alto, CA). DRB0101 in the RSV.5 vector (18) was PCR amplified including 5′ *Xma*I and 3′ *Not*I sites and subcloned into the 5′ *Xma*I and 3′ *Not*I sites of the MCS-B of the pIRES vector: DRB0101 5′ primer, AGTACCCGGGATGGTGTGTCTGAAGCTC; and DRB01013′ primer, TAGTGCGGCCGCTCAGCTCAGGAATCCTGTTG. PCR conditions for both DRA and DRB0101 amplifications were: denature at 94°C for 2 min, denature at 94°C for 1 min, anneal at 60.9°C or 62.9°C (DRA and DRB0101, respectively) for 1 min, extend at 72°C for 3 min (High Fidelity Taq; Roche, Basel, Switzerland); repeat the last three steps 30 times and extend at 72°C for 7 min. The resulting construct is pIRES/DR1 (Fig. 1*A*).

The pLNCX2 retroviral vector (Clontech) was modified to include a linker containing an *Avr*II site in the MCS. To make the linker, equimolar amounts oftheoligonucleotides(5′-GATCTCGAGCTCCTAGGAATTGTTTGGCCGAGGC-3′ and 3′-AGCTCGAGGATCCTTAACAAACCGGCTCCGCCGG5-′) were mixed, heated at 95°C for 5 min, and then incubated at 22°C for 1 h. The resulting linker was ligated to *Bgl*II- and *Not*I-digested pLNCX2. The resulting construct is pLNCX2/*Avr*II.

The DRA-IRES-DRB0101 fragment of the pIRES/DR1 was digested with *Nhe*I and *Not*I and gel purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA) and then ligated to *Avr*II- and *Not*I-digested pLNCX2/*Avr*II. The final MHC class II construct is pLNCX2/DR1 (Fig. 1*A*).

For the pLHCX/CD80(HPH) construct, pLHCX (hygromycin resistance; Clontech) was modified to include a 5′ *Bam*HI site and a 3′ *Hin*dIII site by inserting an oligonucleotide linker between the *Hin*dIII and *Cla*I sites of the MCS. The original *Hin*dIII in the vector was deleted by insertion of the linker. *Xho*I, *Hpa*I, *Avr*II, and *Not*I restriction sites were included in the linker for future cloning purposes. The linker sequence was: L1, 5′-AGCTGCTCGAGTTAACGGATCCTAGGAAGCTTGCGGCCGCAT-3′; and L2, 5′-CGATGCGGCCGCAAGCTTCCTAGGATCCGTTAACTCGAGC-3′.

Human CD80 was excised from the pREP10/B7.1 vector with *Bam*HI and *Hin*dIII and inserted into the modified pLHCX vector using the *Bam*HI and *Hin*dIII sites (Fig. 1*B*).

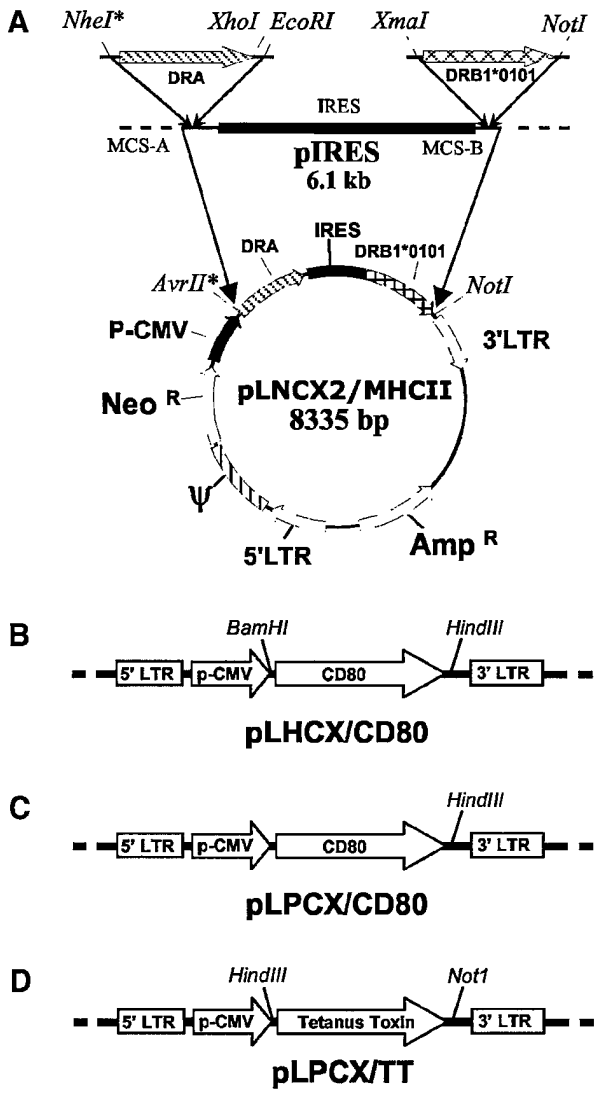

Fig. 1. Retroviral constructs made and used in these studies. *A*, the pLNCX2/MHC II construct contains the DRA and DRB0101 cDNAs flanking an IRES and under the control of the cytomegalovirus (*CMV*) promoter and contains the *G418* resistance gene. *B* and *C*, the pLHCX/CD80 and pLPCX/CD80 constructs encode the human *CD80* gene and contain the hygromycin or puromycin resistance genes, respectively. *D*, the pLPCX/TT construct encodes the TT fragment C gene and contains the puromycin resistance gene. *LTR*, long terminal repeat.

For the pLPCX/CD80 (Puro) construct, the *CD80* gene was excised from pREP10/B7.1 by digestion with *Bgl*II and *Hin*dIII and ligated into pLPCX digested with *Bam*HI and *Hin*dIII. The *Bam*HI and *Bgl*II sites were deleted during this process (Fig. 1*C*).

For the pLPCX/TT construct, TT fragment C DNA was PCR amplified from pCR Blunt (19) to include an ATG start codon and *Hin*dIII site at the 5′ end and a *Bam*HI site at the 3′ end: 5′ primer sequence, CCGCCGAAGCTTGCCACCATGAAAAACCTTGATTGTT; and 3′ primer sequence, CTGTTCGGATCCTTAGTCGTTGGTCCAA. PCR conditions were: denature at 94°C for 5 min, denature at 94°C for 1 min, anneal at 55°C for 1 min, extend at 72°C for 1 min (*Taq*DNA polymerase; Invitrogen); repeat the last three steps 35 times and extend at 72°C for 10 min. The resulting PCR product was inserted into the TA cloning vector, pGEM-T-Easy (Invitrogen). The modified TT fragment C gene was then excised with *Hin*dIII and *Bam*HI and inserted into the mammalian expression vector pCDNA3.1/Zeo(+) (Invitrogen). A *Hin*dIII-*Not*I fragment containing the TT fragment C gene was then excised from pCDNA3.1/Zeo(+) and subcloned into the *Hin*dIII-*Not*I site of the MCS of pLPCX(Puro) to produce the pLPCX/TT vector.

Table 1 *Tumor cell vaccines (transductants) used in these studies*

Cell line	HLA-DRB0101	CD80	TT[a]	Drug selection
SUM/DR1	+			G418[b]
SUM/CD80		+		HPH[c]
SUM/TT			+	Puro[d]
SUM/DR1/CD80	+	+		G418[b] + Puro[d]
SUM/DR1/CD80/TT	+	+	+	G418[b] + HPH[c] + Puro[d]
SUM/DR1/TT	+		+	G418[b] + Puro[d]
SUM/CD80/TT		+	+	HPH[c] + Puro[d]
Mel 202/DR1	+			G418[b]
Mel 202/CD80		+		HPH[e]
Mel 202/DR1/CD80	+	+		G418[b] + Puro[d]

[a] Tetanus toxin fragment C.
[b] 600 μg/ml.
[c] 200 μg/ml.
[d] 0.2 μg/ml.
[e] 75 μg/ml.

Cells. Media for all cell lines contained 1% gentamicin, 1% penicillin/streptomycin (all from BioSource, Rockville, MD), and 2 mM Glutamax (BRL/Life Sciences, Grand Island, NY). All cells and T-cell activation assays were cultured at 37°C in 5% CO_2. SUM159PT was obtained from the Michigan Breast Cell/Tissue Bank[3] and was maintained in Ham's F-12 medium with 10% heat-inactivated FCS (Hyclone, Logan, UT), 1 μg/ml hydrocortisone, and 5 μg/ml insulin (both from Sigma, St. Louis, MO). Mel 202 (20) was grown in RPMI 1640 (BioSource, Rockville, MD) with 10% FCS, 0.01 M HEPES (Invitrogen, Grand Island, NY), and 5×10^{-5} M β-mercaptoethanol (J. T. Baker, Inc., Phillipsburg, NJ). Transductants were grown in the same medium as their parental cells, supplemented with G418 (Sigma), puromycin (Clontech, Palo Alto, CA), or hygromycin (Calbiochem, San Diego, CA; see Table 1 for dosages), depending on their transgenes. Sweig and Jurkat cells were obtained from the American Type Culture Collection and were maintained in Iscove's modified Dulbecco's medium (BioSource) supplemented with 10% fetal clone I (FBP; Hyclone). EBV B cells were grown in RPMI 1640 with 10% FCS and 0.01 M β-mercaptoethanol. Peripheral blood mononuclear cells (PBMCs) were grown in Iscove's modified Dulbecco's medium with 5% human AB serum (Gemini Bio-Products, Woodland, CA). All cell lines and procedures with human materials were approved by the Institutional Review Boards of the participating institutions.

Retrovirus Production. 293T cells (obtained from the Harvard Gene Therapy Institute) were plated in a 6-cm dish at 9×10^5 cells/4 ml of 293T medium [DMEM (BioSource, Rockville, MD), 1% gentamicin, 1% penicillin/streptomycin, 1% Glutamax, and 10% heat-inactivated FCS] and cultured at 37°C. Twenty h later, the growth medium was replaced with 4 ml of 37°C Iscove's modified Dulbecco's medium containing 25 mM HEPES (BioSource), 1% Glutamax, and 10% heat-inactivated FCS. Three h later, the 293T cells were transfected with pLNCX2/DR0101, pLHCX/CD80, pLPCX/CD80, or pLPCX/TT plasmids (8 μg) plus pMD.MLV gag.pol (6 μg) and pMD.G (2 μg) using $CaPO_4$ (21). Twelve to 16 h after transfection, medium was replaced with 293T growth medium containing 10 mM HEPES. Virus was collected 48 h later and either used immediately or stored at −80°C.

Retroviral Transduction. Tumor cells were plated in 6-well plates at 1.2-3 $\times 10^5$ cells/3 ml growth medium/well. Approximately 16 h after plating, when cells were in log phase, growth medium was replaced with 500 μl of viral supernatant mixed with 500 μl of 293T medium containing 4 μg/ml polybrene (Sigma) and 10 mM Hepes. Cells were incubated for 5–6 hrs at 37°C, washed twice with excess PBS and maintained in growth medium for 2 days before adding G418, puromycin, and/or hygromycin.

Peptides, Antibodies, Reagents, and Immunofluorescence. TT p2 peptide $TT_{830-844}$ (QYIKANSKFIGITEL; Ref. 22) was synthesized at the University of Maryland Biopolymer Laboratory. Formaldehyde-inactivated TT was purchased from Accurate Chemical and Scientific Corporation (Westbury, NY).

Monoclonal antibodies [mAbs; HLA-DR-FITC, CD80-phycoerythrin (PE), and anti-TT], streptavidin-PE, FITC-isotype, and PE-isotype controls were purchased from BD PharMingen (San Diego, CA). Biotinylated HLA-DR0101 was purchased from One Lambda, Inc. (Canoga Park, CA). Rat anti-mouse IgG-FITC was purchased from ICN (Costa Mesa, CA), and CD4-FITC, CD8-FITC, and anti-human IgG-FITC were purchased from Miltenyi Biotech (Auburn, CA). Human IgG-FITC was purchased from Cappel (West Chester, PA). Culture supernatants of hybridomas W6/32 (pan HLA-A,B,C), L243 (pan anti-HLA-DR), 28.14.8 (anti-H-$2L^d$, D^b), and PIN1 (anti-Ii) were purified on protein A or protein G affinity columns as described previously (1). Tumor cells and PBMCs were stained for cell surface markers (MHC class I, class II, CD80, CD4, CD8, and immunoglobulin) or fixed and stained for internal markers (Ii, TT) by direct or indirect immunofluorescence as described previously (1, 16). PBMCs were collected by venipuncture from HLA-typed healthy donors and isolated using Histopaque 1077 separation medium as described previously (20). For some experiments, PBMCs were provided by Dr. D. Mann (University of Maryland Baltimore). PBMCs were stored at 1×10^7 cells/ml at −80°C until used.

Western Blots. Western blot analyses were performed as described (14) using 10% SDS-PAGE. Blots were incubated with PIN1.1 mAb (0.003 μg/ml) followed by sheep anti-mouse HRP at a 1:10,000 dilution (Amersham).

Allogeneic T-Cell Activation. Responder PBMCs (1×10^5/well) were cultured in triplicate with 5×10^3 or 1×10^4 irradiated (CS-137 irradiator; Kewaunee Scientific, Statesville, NC) stimulator SUM159PT (50 Gy) or 5×10^5 allogeneic PBMCs (40 Gy) per well in 200 μl/well of culture medium (RPMI, 10% FCS, 1% penicillin/streptomycin, 2 mM Glutamax, and 0.01 M β-mercaptoethanol) in flat-bottomed 96-well microtiter plates (Corning, Inc., Corning, NY). Cells were incubated at 37°C in 5% CO_2 for 6 days and pulsed with [^{3}H]thymidine (2 μCi/well) during the final 18 h, after which the cells were harvested onto glass fiber filter mats using a Packard Micromate 196 cell harvester (Downers Grove, IL). Filter mats were sealed into plastic bags with 5 ml of betaplate scintillation fluid (Perkin-Elmer, Gaithersburg, MD) and counted using a Wallac 1450 Microbeta liquid scintillation counter (Perkin-Elmer). Samples were run in triplicate.

Stimulation Index (SI)

$$= \frac{\text{(cpm of transduced experimental tumor cells + allogeneic PBMC)} - \text{(cpm of transduced tumor cells alone)}}{\text{cpm of allogeneic PBMCs alone}}$$

Responder PBMCs do not express DRB0101.

TT-boosted PBMCs. DRB0101 PBMCs (2×10^7 cells/4 ml/well) were cultured with 1 μg/ml of exogenous TT (Accurate, Westbury, NY) in 6-well tissue culture plates (Corning). After 5 days of culture, nonadherent cells were harvested, washed twice with Iscove's modified Dulbecco's medium, and replated in culture medium with 20 units/ml of recombinant human interleukin 2 (R&D Systems, Minneapolis, MN) at 1×10^6 cells/2 ml/well in 24-well plates (Corning). Remaining nonadherent cells were harvested 7 days later, and live cells were isolated using Histopaque-1077 separation medium. TT-activated, nonadherent cells were maintained in culture medium without exogenous interleukin 2 overnight and used the following day.

Antigen Presentation Assays for Endogenous TT and Exogenous TT Peptides. Irradiated (50 Gy) stimulator cells (1×10^4 or 2.5×10^4 cells/well) were cocultured in triplicate with adherent cell-depleted, TT-primed DRB0101 responder PBMCs (5×10^4 cells/well) in 200 μl/well in flat-bottomed 96-well microtiter plates (Corning). After 2 days of culture, supernatants were collected and assayed by ELISA for IFN-γ according to the manufacturer's instructions (Endogen, Woburn, MA). For the antibody blocking experiments, 1×10^4 stimulator cells were incubated with 10 μg/ml or 12.5 pg/ml of L243 (anti-HLA-DR) or 28.14.8 (isotype-matched irrelevant mAb) in 100 μl/well for 45 min before the addition of responder PBMCs. Values are the averages of triplicate points with their SDs.

For exogenous TT peptide p2 presentation, assays were as for endogenous antigen presentation, except soluble TT peptide p2 (22, 23) was added at the beginning of the 2-day culture period, and antigen-presenting cells not transduced with TT were used.

CD4, CD8, and CD19 Cell Depletions. Adherent cell-depleted, TT-primed PBMCs were depleted for $CD4^+$, $CD8^+$, or $CD19^+$ cells using magnetic beads, LD columns, and the QuadroMACS separation system according to the manufacturer's instructions (Miltenyi Biotech). Purity of depleted fractions was confirmed by flow cytometry.

HLA-DR Nomenclature. The PBMCs used in these studies were HLA typed by PCR; hence, they are known to be HLA-DRB0101. The HLA-DR gene used in these studies was sequenced and identified as HLA-DRB0101 and

[3] Internet address: www.cancer.med.umich.edu/breast_cell/umbnkdb.htm.

is abbreviated as "DR1" in the names of the transductants. The TT p2 peptide has been identified as a DR1-restricted epitope; however, its DR1 subtype is not known.

RESULTS

Construction of Retroviruses Encoding HLA-DRα Plus HLA-DRβ, CD80, and TT Fragment C. To generate human tumor cells expressing high levels of MHC class II molecules, retroviruses encoding HLA-DRα plus HLA-DRβ genes have been generated. The HLA-DRB0101 allele was selected because it is one of the more common alleles in the Caucasian population and is a frequently used restriction element (24, 25). A novel bicistronic retroviral vector that drives coordinate expression of approximately equimolar amounts of HLA-DRα and HLA-DRβ was developed using the pLNCX2(neo) retroviral backbone. DRα and DRβ0101 cDNAs (18) were cloned upstream and downstream, respectively, of the internal ribosomal entry site (IRES) of the vector pIRES. The DRA-pIRES-DRB segment was then excised from the pIRES vector and ligated into the pLNCX plasmid to yield the pLNCX/DRβ1 plasmid (Fig. 1*A*). This construct will produce a single-chain mRNA driven by the cytomegalovirus promoter in which DRα is translated by a CAP-dependent mechanism and DRβ is translated via the IRES in a CAP-independent manner.

Because of the critical role of costimulatory molecules in the activation of naive T cells (26), we have also generated retroviral plasmids encoding human CD80 (hCD80). The *hCD80* gene was excised from the pREP10/B7.1 plasmid and ligated into the retroviral vector pLHCX(HPH) or pLPCX(Puro) to form the pLHCX/CD80 (Fig. 1*B*) or pLPCX/CD80 (Fig. 1*C*) plasmids, respectively.

To monitor presentation of endogenously synthesized antigen, a retroviral plasmid encoding the TT fragment C was generated. The TT fragment C gene was excised from the pCR Blunt plasmid, an ATG start codon was inserted at its 5′ end, and the resulting construct was ligated into the pLPCX(Puro) vector to form the pLPCX/TT retroviral plasmid (Fig. 1*D*). All retroviral plasmids were packaged in 293T cells, and supernatants containing infectious retroviruses were harvested and used to transduce target tumor cells.

Transduced Human Tumor Cells Express Cell Surface HLA-DRB0101 and CD80 and Internal TT. The human ocular melanoma cell line Mel 202 and the mammary carcinoma cell line SUM159PT were transduced with different combinations of the pLNCX2/DR1, pLHCX/CD80, pLPCX/CD80, and pLPCX/TT retroviruses. The resulting transductants are shown in Table 1. SUM159PT and Mel 202 tumors were chosen because they do not constitutively express MHC class II molecules and hence should not express Ii, which we have shown previously inhibits presentation of MHC class II-restricted endogenous antigens (14, 17). To assess the magnitude and stability of transgene expression, transductants were tested by immunofluorescence and flow cytometry 1 week after being placed on drug selection (see Table 1 for drug selection conditions for each transductant line) and intermittently for 6 months thereafter. As shown in Fig. 2, Mel 202 and SUM159PT transductants express high levels of cell surface HLA-DR (L243 mAb), CD80 (CD80-PE mAb), and internal TT (polyclonal anti-TT ab), as measured at 6 months after transduction. HLA-DR-expressing Mel 202 and SUM159PT cells were also biotinylated, and the cell extracts were immunoprecipitated with anti-HLA-DR mAbs to assure proper structural conformation of cell surface-expressed, transduced class II molecules. Both lines displayed high levels of SDS-stable MHC class II $\alpha\beta$ dimers, indicating proper conformation and peptide binding.[4] The parental lines and transductants were also stained for MHC class I molecules (W6/32 mAb). All lines showed strong class I expression, with transductants displaying levels roughly comparable with their parental lines (data not shown).

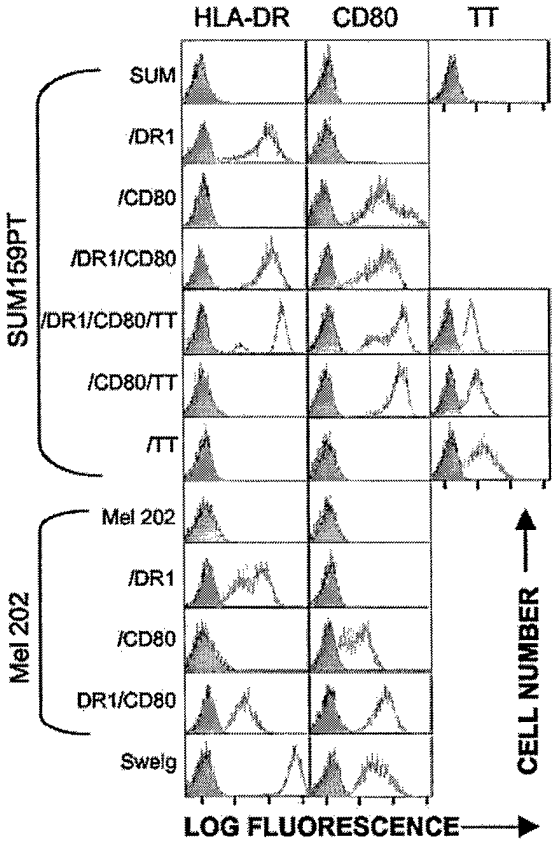

Fig. 2. SUM159PT and Mel 202 transductants express MHC class II HLA-DR and CD80 on the cell surface and TT internally. Live transductants were stained by direct immunofluorescence for plasma membrane HLA-DR (L243-FITC) or CD80 (CD80-PE). Fixed cells were stained by indirect immunofluorescence for internal TT (TT mAb plus fluorescent conjugate). *Gray peaks* denote staining with fluorescent conjugate alone or isotype control; *white peaks* represent staining with directly coupled primary antibody or primary antibody plus fluorescent conjugate. These data are from one of three to five independent experiments.

To ascertain that the MHC class II expression is allele specific, SUM/DR1 and SUM/DR1/CD80 cells were stained for cell surface expression of HLA-DR1 using the HLA-DR1-specific mAb. As shown in Fig. 3, pLNCX2/DR1-transduced SUM cells express high levels of DR1 and only stain at background levels with an irrelevant HLA-DR2-specific mAb. Therefore, SUM/DR1/CD80, SUM/DR1, SUM/CD80, SUM/DR1/CD80/TT, Mel 202/DR1, Mel 202/CD80, and Mel 202/DR1/CD80 transductants express high levels of the transduced *HLA-DR, CD80,* and/or *TT* genes as measured by antibody reactivity and immunofluorescence.

SUM159PT and Mel 202 Cells Do Not Express Invariant Chain. Because coexpression of Ii inhibits endogenous antigen presentation by MHC class II vaccine cells (14, 17), SUM159PT and Mel 202 cells were tested to ascertain that they do not express Ii. Cells were permeabilized, stained with the Ii-specific mAb PIN-1, and analyzed by flow cytometry. As shown in Fig. 4*A,* neither tumor line contains Ii, whereas the human B cell line, Sweig, which constitutively expresses Ii, is strongly positive. To further confirm the absence of Ii, detergent extracts of SUM159PT, Mel 202, Ii-positive Sweig, and Ii-negative Jurkat cells were electrophoresed by SDS-PAGE and analyzed by Western blotting for Ii expression. As shown in Fig. 4*B,* neither SUM159PT, Mel 202, Mel 202/DR1/CD80, nor SUM/DR1/

[4] V. Clements, unpublished results.

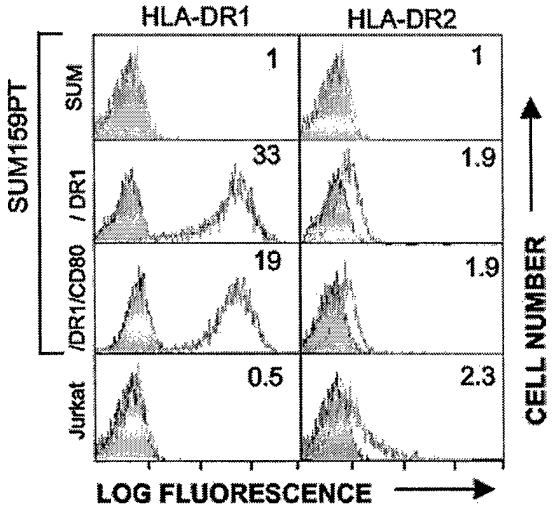

Fig. 3. SUM/DR1 and SUM/DR1/CD80 transductants express HLA-DR1 at the cell surface. Live cells were stained by indirect immunofluorescence for plasma membrane HLA-DR1 (mAb HLA-DR1 biotin) or with an irrelevant Ab (HLA-DR2-biotin) plus an avidin-PE conjugate. Jurkat is a DR1$^-$ cell line. *Gray peaks* denote staining with fluorescent conjugate without primary antibody; *white peaks* represent staining with primary antibody plus fluorescent conjugate. Numbers in the *upper right-hand corner* of each profile are the mean channel fluorescence for the antibody stained peak. These data are from one of two to five independent experiments.

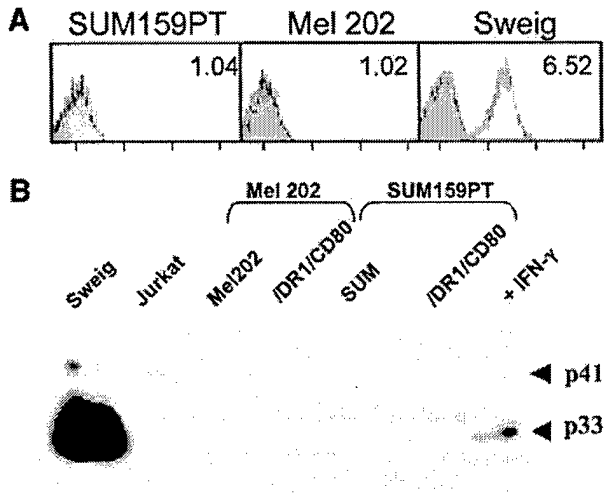

Fig. 4. Sum159PT and Mel 202 cells do not express Ii chain. *A*, fixed cells were stained by indirect immunofluorescence for Ii (mAb PIN1.1; *white peaks*) or with fluorescent conjugate alone (*gray peaks*). These data are representative of three independent experiments. *B*, uninduced or IFN-γ-treated (+ *IFN*γ) cells were detergent lysed, electrophoresed on 10% SDS-PAGE gels under reducing conditions, and transferred to nitrocellulose. Blots were stained for Ii with the mAb PIN1.1. Sweig and Jurkat cells are Ii$^+$ and Ii$^-$ cell lines, respectively. These data are from one of two to three independent experiments.

CD80 cells contain detectable Ii, although Ii expression is inducible in SUM159PT cells by a 48-h treatment with IFN-γ. Therefore, SUM159PT and Mel 202 tumor cells do not constitutively express Ii; therefore, Ii will not be present in the transduced vaccine cells to inhibit binding and presentation of endogenously synthesized peptides.

HLA-DRB0101 Transductants Stimulate HLA-DR Allogeneic PBMCs. Coculture of cells expressing functional HLA-DR molecules with allogeneic CD4$^+$ T lymphocytes results in T-cell proliferation (27). Therefore, to determine whether the HLA-DRB0101 molecules expressed by the transduced tumor cell vaccines are functional, we cocultured the various transductants with allogeneic PBMCs. Responder non-HLA-DRB0101 PBMCs were mixed with various numbers of irradiated transductants, and proliferation was assessed by measuring the SI at the end of 6 days of culture. Irradiated allogeneic PBMCs were used as a positive control. As shown in Fig. 5*A*, SUM/DR1/CD80 cells induce high SI, whereas SUM, SUM/CD80, or SUM/DR1 transductants produce only background levels. Therefore, the cell-based vaccines activate allogeneic PBMCs, provided they coexpress DRB0101 and CD80.

Transduced Tumor Cells Present an HLA-DR1-restricted TT Peptide. TT peptide p2 is an HLA-DR1-restricted epitope (22). If the HLA-DRB0101 molecules of the transductants are properly conformed and functional, when pulsed with the TT p2 peptide, the transductants should activate TT-specific HLA-DRB0101 lymphocytes. Because the TT-specific CD4$^+$ T-cell precursor frequency in peripheral blood of the DRB0101 donor was low (data not shown), the HLA-DRB0101 PBMCs were boosted *in vitro* with TT to expand the number of TT-reactive T cells. TT-boosted PBMCs were incubated at various ratios with tumor cell transductants pulsed with various quantities of TT p2 peptide to determine whether the transductants present this HLA-DR1-restricted epitope. T-cell activation was assessed by measuring IFN-γ release. As shown in Fig. 5*B*, SUM/DR1/CD80 tumor cells activate the TT-specific T cells as or more efficiently than EBV-transformed HLA-DR1 B cells (DR1-EBV B cells), whereas HLA-DR1-negative parental SUM cells do not activate. Therefore, SUM/DR1/CD80 tumor cells are effective APCs for an HLA-DR1-restricted epitope, further demonstrating that the transduced MHC class II molecules are functional.

HLA-DR1/CD80 Tumor Cell Transductants Present Endogenous TT and Activate TT-specific T Lymphocytes. We have generated the DR1/CD80 transductants to use as cancer vaccines to immunize patients and activate their T lymphocytes to tumor-encoded

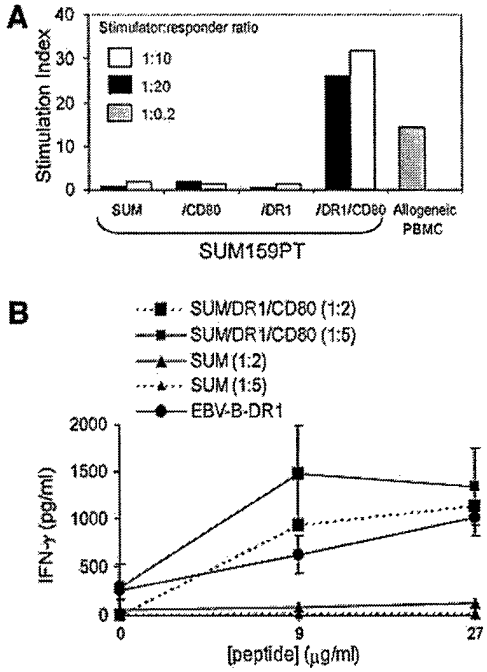

Fig. 5. SUM/DR1/CD80 cells induce proliferation of allogeneic T cells and present a DR1-restricted peptide to DRB0101 PBMC. *A*, irradiated SUM transductants expressing HLA-DRB0101 and/or CD80 or allogeneic PBMCs were cocultured with non-DRB0101 PBMCs at various ratios of APCs to responder lymphocytes. Proliferation was assessed by measuring the SI after 6 days of culture. These data are from one of three independent experiments. *B*, SUM, SUM/DR1/CD80, or DRB0101-expressing EBV B cells were pulsed with the DR1-restricted TT peptide, p2, and cocultured with TT-primed DRB0101 PBMCs at various ratios of APCs to PBMCs. Supernatants were harvested after 2 days of culture and tested by ELISA for IFN-γ. These data are from one of three independent experiments. *Bars*, SD.

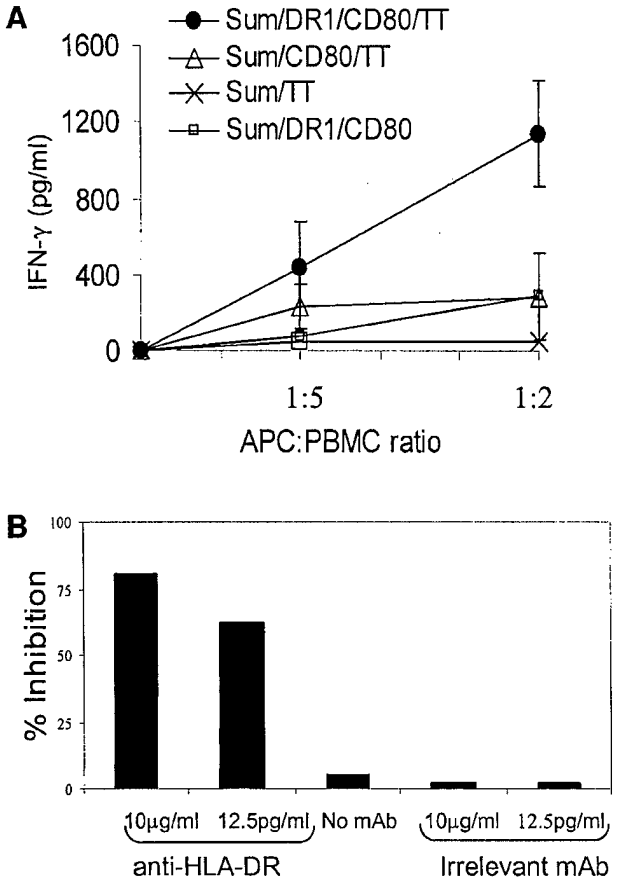

Fig. 6. SUM/DR1/CD80/TT cells activate HLA-DR-restricted DRB0101 PBMCs to tumor-encoded TT. *A,* irradiated SUM transductants were cocultured with TT-primed DRB0101 PBMCs at various ratios of APCs to responder PBMCs. Supernatants were harvested after 2 days of culture and tested by ELISA for IFN-γ. These data are from one of three independent experiments; *bars,* SD. *B,* HLA-DR-specific (L243) or irrelevant (28-14-8) mAb was added to culture wells containing irradiated SUM/DR1/CD80/TT cells before addition of TT-primed responder DRB0101 PBMCs at a ratio of 1:2 APCs to responder cells. Supernatants were harvested after 2 days of culture and tested by ELISA for IFN-γ. These data are from one of two independent experiments.

tumor peptides. To achieve this goal, the transductants must not only express functional HLA-DR molecules, but the DR molecules must also bind and present endogenously synthesized tumor peptides. To determine whether the transductants have this capability, we tested SUM/DR1/CD80/TT cells as APCs for endogenously encoded TT. Because the TT construct does not contain a signal sequence, TT protein will reside in the cytoplasm and serve as a "model" tumor antigen for a cytoplasmically localized tumor antigen.

Adherent cell-depleted HLA-DRB0101 PBMCs were boosted *in vitro* with TT as per the experiment of Fig. 5*B* and cocultured at various ratios with irradiated transduced SUM cells. Activation was assessed by measuring IFN-γ release. As shown in Fig. 6*A,* SUM/DR1/CD80/TT tumor cells activate a potent T-cell response, whereas SUM transductants without DRB0101 (SUM/CD80/TT), without TT (SUM/DR1/CD80), or without DRB0101 and CD80 (SUM/TT) do not activate. Because SUM/TT and SUM/CD80/TT cells do not activate, TT is not being released into the culture medium and being presented by other APCs in the PBMC population. Therefore, tumor cells transduced with *HLA-DRB0101, CD80,* and *TT* genes are effective APCs for endogenously encoded molecules.

To further analyze whether the presentation of endogenous TT is DR1 restricted, anti-HLA-DR mAb (L243) was added at various concentrations at the beginning of the assay. As shown in Fig. 6*B,* in the presence of the highest dose of antibody, T-cell activation is inhibited >80%, whereas an irrelevant isotype-matched mouse H-2L^d-specific mAb does not inhibit.

DR1/CD80/TT Tumor Cells Activate CD4+ T Lymphocytes. To identify the PBMCs that are specifically activated by the vaccine cells, adherent cell-depleted, TT-primed DRB0101 PBMCs were depleted for CD4+ or CD8+ T cells or for B cells and then used as responding cells in antigen presentation assays with SUM/DR1/CD80/TT transductants. T and B cells were depleted by magnetic bead separation. To ascertain the efficiency of the depletions, PBMCs before and after depletion were tested by flow cytometry for the percentage of CD4+, CD8+, and immunoglobulin+ (B) cells. As shown in Fig. 7*A,* antibody depletion eliminated 98–99% of the target lymphocytes. The relatively high percentage of CD4+ T cells and low percentage of CD8+ T cells in the undepleted, TT-boosted population probably reflects the preferential activation of CD4+ T cells during the *in vitro* boosting process.

After T- and B-cell depletion, the resulting PBMCs were cocultured with irradiated vaccine cells and endogenous TT presentation assessed by ELISA. As shown in Fig. 7*B,* CD4-depleted PBMCs stimulated with SUM159/DR1/CD80/TT vaccine cells are not activated, as measured by IFN-γ release. In contrast, CD8-depletion did not affect IFN-γ release. Likewise, depletion of CD19+ cells did not affect IFN-γ release, demonstrating that cross-priming by B cells is not occurring. Stimulation of undepleted PBMCs with SUM159/DR1/CD80 APCs also did not cause IFN-γ release, demonstrating that

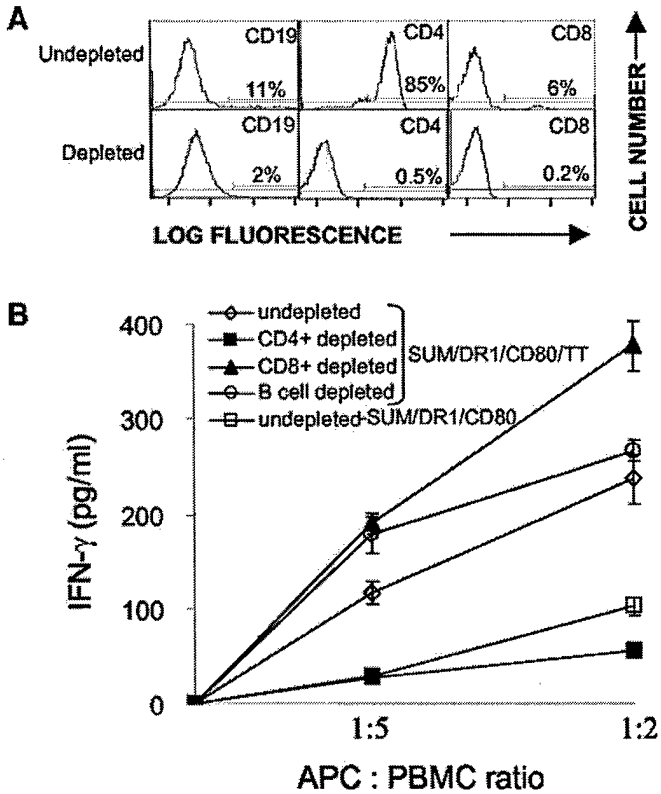

Fig. 7. SUM/DR1/CD80/TT cells activate CD4+ T lymphocytes to tumor-encoded antigen. *A,* DRB0101 PBMCs were primed *in vitro* with TT, and separate aliquots were depleted for CD4+, CD8+, or CD19+ cells. The resulting cells were stained by direct immunofluorescence for these populations. Values in the *lower right-hand corners* of each profile represent the percentage of the indicated cells. *B,* irradiated SUM/DR1/CD80/TT or SUM/DR1/CD80/transductants were cocultured with CD4, CD8, or CD19-depleted, or not depleted, TT-primed DRB0101 PBMCs at various ratios of APCs to responder PBMCs. Supernatants were harvested after 2 days of culture and tested by ELISA for IFN-γ. These data are from one of two to four independent experiments; *bars,* SD.

PBMC activation is TT specific. Therefore, $CD4^+$ PBMCs are activated by the vaccine cells, and the activation is mediated by direct presentation of endogenously synthesized TT by the genetically modified tumor cell transductants.

DISCUSSION

Recent animal studies and some clinical trials have indicated that the use of genetically engineered tumor cells as vaccines may have therapeutic efficacy for the treatment of cancer (28–30). Parallel studies have recognized the critical role played by $CD4^+$ T cells in orchestrating the host immune response against cancer and have developed methods to activate $CD4^+$ T cells (2–5, 31–33). Because $CD4^+$ T cells play a central role in enhancing antitumor immunity, our laboratory has focused on facilitating the activation of these cells. We have hypothesized that tumor cells that constitutively express MHC class I molecules do not contain Ii and are genetically modified to express syngeneic MHC class II molecules, and costimulatory molecules will function as APCs for endogenously synthesized MHC class I- and class II-restricted tumor antigen epitopes. If used as immunogens in tumor-bearing individuals, such cells will serve as "vaccines" to activate tumor-specific $CD4^+$ and $CD8^+$ T lymphocytes that will facilitate regression of wild-type tumor (2, 6). Because the efficacy of these vaccines against wild-type primary tumors and experimental and spontaneous metastatic disease has been demonstrated in multiple mouse models (8, 10, 11, 34, 35), the goal of this study was to translate this strategy for clinical use.

Activation of tumor-specific $CD4^+$ T cells by the cell-based vaccines is based on the supposition that the MHC class II molecules of the vaccine cells bind peptides synthesized within the tumor cells and directly present these peptides to $CD4^+$ T lymphocytes. This mode of presentation is different from that of professional APCs that typically bind peptides derived from endocytosed, exogenously synthesized antigens (36). This fundamental difference is attributable to the absence of the MHC class II-associated accessory molecule, Ii, in the vaccine cells. If APCs express Ii, Ii binds to newly synthesized MHC class II molecules, thereby preventing the binding of endogenously derived peptides and favoring the binding of exogenously synthesized peptides (37). However, in the absence of Ii, MHC class II molecules bind peptides derived from endogenously synthesized antigens (14). Because the MHC class II and Ii genes are coordinately regulated and coordinately induced by IFN-γ (38), professional APCs and tumor cells that constitutively express MHC class II genes and/or are induced by IFN-γ are unlikely to be APCs for endogenously synthesized tumor antigens. Studies with Ii^+ and Ii^- MHC class II^+ tumor cells support this concept and demonstrate that the most efficacious vaccines are MHC class II^+Ii^- (14, 16, 17).

Early studies suggested that expression of MHC class II molecules without coexpression of Ii produces reduced levels of class II molecules that are improperly conformed and unable to function as antigen presentation elements (39–41). More recent studies have demonstrated that the Ii dependency of MHC class II molecules is allele specific (42, 43), and that many MHC class II alleles do not require Ii expression for stability or antigen presentation function (44). The studies reported here demonstrating efficient antigen presentation by MHC class II^+Ii^- tumor cell vaccines add HLA-DR0101 to the list of MHC class II alleles whose expression and function are independent of Ii coexpression.

In addition to the absence of Ii for maximal vaccine efficacy, the studies reported here demonstrate that optimal vaccine activity requires coexpression of CD80 for delivery of a costimulatory signal. This observation agrees with extensive mouse and human studies showing the requirement for costimulation for optimal T-cell activation (reviewed in Ref. 26), as well as many studies that showed that CD80 expression facilitates tumor rejection (45–47).

Several lines of evidence support the hypothesis that the MHC class II tumor cell-based vaccines activate $CD4^+$ T cells by direct antigen presentation of endogenously encoded tumor antigens, rather than by cross-priming or indirect presentation via host-derived APCs, as suggested by other investigators for other cell-based vaccines and/or tumor cells (48–50):

(*a*) If tumor-encoded antigens were presented by host-derived APCs such as B cells or other APCs in the PBMCs, then SUM/DR1/TT, SUM/TT, and SUM/CD80/TT cell lines should be just as effective APCs as are SUM/DR1/CD80/TT. However, only SUM/DR1/CD80/TT vaccine cells activate PBMCs.

(*b*) If professional APCs, rather than the tumor cell vaccines, are the relevant APCs, then removal of these professional APCs should eliminate T-cell activation. However, adherent cells (including dendritic cells and macrophages) are routinely removed from the PBMCs before their coculture with vaccine cells, and in some experiments, $CD19^+$ B cells were also removed without affecting T-cell activation.

(*c*) Extensive *in vivo* studies using genetically marked vaccine cells conclusively demonstrated that the vaccine cells directly activate T lymphocytes (12–14). Therefore, it is unlikely that vaccine efficacy is attributable to leakage of tumor antigen, resulting in endocytosis by professional APCs for presentation by cross-priming.

The vaccines described here are based on the premise that tumor cells will be destroyed by $CD8^+$ T cells with help from $CD4^+$ T cells. Tumor-specific $CD8^+$ T cells could be activated either by interacting with MHC class I/peptide complexes of the genetically modified vaccine cells or by cross-presentation of class I-restricted epitopes by professional APCs. In either case, the activated $CD8^+$ T cells would be specific for MHC class I-restricted tumor peptides and for wild-type tumor cells. Although the vaccines described here are potent activators of $CD4^+$ T cells, vaccine cell expression of a MHC class I allele shared with the patient's lymphocytes may facilitate an even stronger immune response by capitalizing on the close proximity of $CD4^+$ and $CD8^+$ T cells during their activation. A MHC class I allele could be expressed in the vaccines by retroviral transduction. Alternatively, for an allele such as HLA-A2, which is expressed by approximately 50% of the Caucasian population, an $HLA\text{-}A2^+$ tumor cell line could be used as the "base" vaccine. Additional experiments assessing activation of $CD8^+$ T cells by the vaccines generated in this study *versus* MHC class I-matched or -engineered vaccines will be necessary to address this issue.

A significant technical obstacle in generating the MHC class II cell-based vaccines has been to routinely achieve high level expression of the desired MHC class II alleles in human tumor cells. Because many human tumor cells and cell lines can be problematic to maintain in culture, standard transfection and electroporation techniques did not result in reproducible class II expression.[5] In contrast, transduction using a bicistronic retrovirus encoding the DRα and DRβ chain genes separated by an IRES routinely yielded high-level HLA-DR expression in a high proportion of transductants. The efficiency of the current retroviruses appears to be attributable to the placement of the DRα and DRβ genes flanking the IRES, because a previous study using a retroviral construct encoding pig DQα and DQβ genes run off of separate promoters and without an IRES produced only low-level, DQ-expressing cells (51). It is likely the IRES construct will be universally useful, because similar retroviruses encoding other HLA-DR alleles also reproducibly yield high-level MHC class II expression in additional human tumor lines.[6]

The potency of the MHC class II vaccines for activating $CD4^+$ T cells

[5] S. Dissanayake and J. Bosch, unpublished results.

[6] J. Thompson and M. Pohl, unpublished results.

to tumor-encoded antigens suggests that these vaccines may have therapeutic efficacy for cancer patients. For example, the cell-based vaccines could be administered *in vivo* to patients with disseminated metastatic disease. Alternatively, they could be used *ex vivo* to activate patients' T cells for subsequent adoptive transfer. In either case, these vaccines provide a novel and potent approach for activating tumor-specific $CD4^+$ T cells and merit further clinical development and testing.

ACKNOWLEDGMENTS

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